

THE EFFECTS OF THE EXTRACT OF RUBAIYAT, AN
OKLAHOMA GRAPE VARIETY, AND
RESVERATROL ON CELLULAR PROLIFERATION
AND APOPTOSIS OF MCF-7 HUMAN BREAST
CANCER CELLS

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CHAPTER I

INTRODUCTION

Cancer is the second most common cause of death in the U.S. and accounts for one of every four deaths (American Cancer Society, Surveillance Research, 2008). In 2007, the overall estimated cost of cancer according to the National Institutes of Health was \$219.2 billion (American Cancer Society, Surveillance Research, 2008).

Breast cancer is the most frequently diagnosed cancer in women, excluding skin cancers, and ranks second as cause of cancer death in women (American Cancer Society, Surveillance Research, 2008). More than 200,000 new breast cancer cases are diagnosed yearly with more than 40,000 women dying annually from the disease (Lopez-Otin et al., 1998). There has been a slight decline in breast cancer incidence beginning around 2000 which may be a result of decreased screening and decreased use of hormone replacement therapy. Death rates in women from breast cancer have also decreased since 1990. This is said to be a result of progress in earlier detection and improved treatment (American Cancer Society, Surveillance Research, 2008).

Although the pathogenesis of breast cancer is still not well understood, age and race are known to play an important role in determining breast cancer risk (Smigal et al., 2006). There have been certain factors that have had long-standing implications in breast cancer such as the use of steroid hormones (Lopez-Otin et al., 1998). Breast cancer incidence can be attributed to age, gender, genetic predisposition, and ethnicity (American Cancer Society, Surveillance Research, 2008). Hormonal effects and environmental factors also appear to play a major role in the development and progression of the disease (Tsubura et al., 2005, Colditz et al., 1995, Adami et al., 1998).

In the pathogenesis and progression of breast cancer, there are various courses of treatment, such as radiation, chemotherapy, and surgery which have varying degrees of side effects. Many side effects of conventional therapies include loss of appetite, weight, loss of hair, nausea and vomiting just to name a few. Tamoxifen, a non-steroidal anti-estrogen, is a pharmacological option commonly used to treat all stages of breast cancer. The drug was first approved in 1973 for the treatment of breast cancer as a chemopreventive agent. Due to a known link between estrogen and the initiation and growth of some breast cancers, tamoxifen was also tested as a potential treatment for advanced breast cancer in postmenopausal women (Smigal et al., 2006, Jordan et al., 2003).

Today, chemoprevention agents are the most promising non-toxic option for reducing cancer incidence by possibly exerting their effects either by blocking or metabolizing carcinogens or by inhibiting tumor cell growth. They have recently been used in cancer treatment in combination with conventional chemotherapeutics or radiotherapy (Sarkar et al., 2007).

Dietary factors are widely believed to play an important role in determining the risk of many cancers, including breast cancer. A diet rich in fruits and vegetables is rich in antioxidants which have been shown to have beneficial roles in cancer prevention (Lopez-Otin et al., 1998). Research supports the role of reactive oxygen species in cancer and that dietary antioxidants as well as endogenous antioxidants neutralize or trap reactive oxygen species, thereby acting as cancer preventive agents (Borek, 2004 A, Steinmetz et al., 1996). Dietary modifications such as increasing fruit and vegetable intake are favored over pharmacological options due to the costs associated with pharmaceuticals and the side effects from conventional therapies.

A fruit that may play an important role in the inhibition and prevention of breast cancer are grapes which are high in polyphenols. Phenolic compounds found in grapes such as resveratrol have been shown to act as antioxidants which can scavenge free radicals and delay or prevent cancer development (Tsubura et al., 2005, Jang et al., 1997, Nakagawa et al., 2001, Joe et al., 2002, Kim, et al., 2004). The antioxidant activity of red grape proanthocyanidins has been demonstrated by the inhibition of aromatase enzyme activity and growth of cancer cells, and preventing disease in various animal models (Eng et al., 2003, Agarwal et al., 2000, Zhao et al., 1999).

Polyphenolic content of grapes vary by variety and growth conditions (Shi et al., 2003). To our knowledge, there is limited research on grape varieties grown in Oklahoma. Although there have been several studies that have investigated the anti-cancer properties of resveratrol and grape seed extract (Singletary, Jung, and Giusti, 2007, Mantena et al., 2006, Kim, Hall, and Smith et al., 2004, Laux et al., 2004, Pozo-Guisado et al., 2005), to our knowledge there is no study conducted of Rubaiyat, an

Oklahoma red grape variety. Moreover, the Rubaiyat grape variety was chosen due to its relatively high polyphenol content. Research on the disease prevention properties of Oklahoma grapes will help Oklahoma grape growers, a relatively new and expanding industry, promote their product. Studying the anti-proliferative properties of an Oklahoma grape variety will help promote the importance of grape growing in the state.

The *hypothesis* of this study is that the polyphenol-rich grape extract of Rubaiyat grapes possess anti-proliferative properties similar to that of resveratrol. The *specific aim* of this study is to compare the effect of Rubaiyat polyphenolic-rich grape extract to that of resveratrol in (a) inhibiting cell proliferation and (b) inducing apoptosis in human breast cancer MCF-7 cell line. Moreover, we will try to begin to explore the anti-proliferative mechanism of action of Rubaiyat polyphenolic-rich grape extract by investigating the relative mRNA gene expression of apoptotic genes, Bax and Bcl-2.

The study has several *limitations*:

- (a) Our study utilized an *in vitro* system which behaves differently than *in vivo* systems. Animal and human studies are needed to confirm the findings of our study.
- (b) The use of phenolic-rich grape extract has different effects compared to that of whole grapes. Although Rubaiyat grape variety is high in polyphenol content, there are other components of grapes that may promote the inhibition of cellular proliferation and induction of apoptosis. Isolating the phenolic-rich grape extract may have eliminated other compounds that may work together with polyphenols to inhibit breast cancer. Other compounds in Rubaiyat grapes alongside the polyphenols may present other potential anti-cancer effects. Although pure grape extracts can give definitive results *in vitro* or in animal studies, research on whole grapes is also as important as humans

generally consume whole grapes. Human studies are warranted for the determination of the effects of the whole grape on breast cancer inhibition and prevention.

(c) We did not attempt to isolate phenolic compounds from the different parts of grapes (pulp, seed, or skin). Zhao et al., (1999) have demonstrated different amounts and kinds of polyphenols in these different parts of grapes. During extraction of polyphenols, whole grapes were used.

CHAPTER II

REVIEW OF LITERATURE

I. Breast Cancer

A. Prevalence

According to the American Cancer Society (ACS), breast cancer accounts for more than 1 in 4 cancers diagnosed in U.S. women (ACS, Breast Cancer Facts & Figures, 2007). However, the incidence rates for breast cancer decreased by 3.5% per year between 2001-2004 and the mortality rate decreased by 2.2% annually from 1990-2004 (ACS, Breast Cancer Facts & Figures, 2007). This decline, while more prevalent among the younger age groups, has been attributed to improvements in treatment and early detection (ACS, Breast Cancer Facts & Figures, 2007).

The incidence and death rates for breast cancer tend to increase with age; 95% of new cases and 97% of deaths occurred in women 40 and older during 2000-2004 (ACS, Breast Cancer Facts & Figures, 2007). During these same years, women 20-24 years of age had the lowest rate of incidence (1.4 cases per 100,000 women) while 464.8 cases per 100,000 were observed in women aged 75-79 years. In the years 2000-2004, the median age at the time of diagnosis of breast cancer was 61 years old (ACS, Breast Cancer Facts & Figures, 2007).

In addition to age, other known risk factors for breast cancer include gender, genetic predisposition, and ethnicity. Breast cancer differs among ethnicities with highest incidence in White women followed by African Americans, Asian Americans/Pacific Islanders, Hispanics/Latinas, and American Indians/Alaska Natives (Jemal et al., 2006, Smigal et al., 2006). Whites and African American women are two populations more likely to die from breast cancer at every age, however, death rates are lower among white women than African American women (ACS, 2007). However, from 1992-2004, American Indians/Alaska Natives showed decreased incidence while there was no significant change among Asian Americans/Pacific Islanders or Hispanics/Latinas (ACS, 2007).

Epidemiological studies show that breast cancer is 5-fold higher in Western countries than in some Asian countries (Adami et al., 1998). The higher breast cancer incidence rate among Whites is thought to reflect a combination of factors that affect diagnosis, such as a more frequent mammography in White women, and factors that affect disease risk such as later age at first birth and greater use of hormone replacement therapy among Whites than African American women (Jemal et al., 2006, Smigal et al., 2006).

B. Etiology and pathogenesis

The etiology of human breast cancer is largely unknown. Several factors have been suggested to play a role in the development and progression of breast cancer. These factors include age, gender, genetic susceptibility, ethnicity, chronic inflammation, hormonal effects, and environmental factors such as alcohol consumption and smoking

(Smigal et al., 2006, Valko et al., 2004, Colditz et al., 1995). However, known genetic risk factors are only present in a small number of breast cancer cases. Human breast cancer occurs with 100-fold greater incidence in females than in males (Tsubura et al., 2005, Colditz and Frazier, 1995, Adami et al., 1998), who are rarely affected which is suggested to be due to androgenic dominance over estrogens which keeps breast underdeveloped throughout life (Lopez-Otin et al., 1998). Early age of menarche, late age of menopause and late age of full-term pregnancy are associated with increased breast cancer risk. Such associations are suggested to be due to estrogens which play an important role in the etiology of breast cancer (Tsubura et al., 2005).

Generation of reactive oxygen species (ROS) have also been implicated in the development of many chronic diseases including breast cancer. Many cellular processes of the body produce ROS such as through leakage from the mitochondria and electron transport chain, two very important biological processes for normal functioning of the body (Valko et al., 2004). At normal concentrations, ROS can act as a defense mechanism against foreign agents to rid the body of toxic substances; however, it can also act as disease causing agents if present in excess amounts. It has been estimated that anywhere from 10,000-20,000 free radicals attack each cell of the body every day and varies from person to person (Valko et al., 2004). ROS can cause genetic mutations and chromosomal rearrangements that can lead to cancer. Once cancer occurs, oxidative stress increases the progression of the disease (Valko et al., 2004, Nishikawa et al., 2008). Endogenous antioxidants decrease with age, as does DNA repair capacity; exposure to radiation and to anticancer drugs that produce free radicals further decreases cellular and blood levels of antioxidants (Borek, 2004 A, Borek, 2004 B).

Different metabolic pathways are affected during carcinogenesis. Amundadottir and Leder (1998) analyzed the activation and requirement for signal transduction pathways in mammary gland tumors initiated by different oncogenes. Transgenic mice over-expressing oncogenes that encode for specific growth factors, growth factor receptors, and transcription factors involved in carcinogenesis showed that two signal transduction pathways are activated in an oncogenic specific manner in mammary tumors and their derived cell lines. These pathways include the Erk/MAP kinase pathway in *neu* and v-Ha-*ras* initiated mammary tumors and PI 3-kinase activity in *TGF α* , *heregulin/NDF* and *neu* initiated tumor cell lines. This specificity suggests that oncogenes might use different signaling pathways in order to transform mammary epithelial cells (Amundadottir and Leder, 1998).

In an *in vitro* study of human MCF-7 and T47D breast cancer cell lines, Gomez and colleagues (2007) showed that spliced human X-box binding protein-1 (XBP1 (S)), is stably over expressed in these cell lines and does not require estrogen for cell growth and is resistant to certain breast cancer chemotherapy drugs. It was also found that XBP1 (S) overexpression alters the expression of several apoptotic and cell cycle genes and promotes cell survival. The antiapoptosis gene, Bcl2, is one of the several up-regulated genes by XBP1 (S) in MCF-7 cells (Gomez et al., 2007).

In another *in vivo* study, Liu et al. (2007) investigated the role of erbB3 in erbB2 RTK activity and erbB2-mediated tumorigenesis (proteins involved in tumorigenesis) and tamoxifen resistance. The results suggest that kinase activity of erbB2 phosphorylates erbB3 and leads to the subsequent activation of downstream signaling of other kinases, for example PI-3K/Akt. The downregulation of erbB3 disrupts heterodimer formation

and results in the inactivation of downstream signaling. The downregulation of these kinases may result in less resistance to tamoxifen by breast cancer cells (Liu et al., 2007).

Investigators have researched and proposed many signal transduction pathways and cascades involved in the development and progression of breast cancer. There have been studies conducted on chemotherapy resistant breast cancers. This is a vast field of research that deserves and continues to receive lots of attention. The possibilities of preventing and treating the often times fatal disease as well as reducing chemotherapy resistance is of great importance to society.

Jourdain and Martinou (2009) report that the Bcl-2 family of proteins is pro-apoptotic proteins which permeabilize the mitochondria during apoptosis by integrating and conveying death signals to the mitochondria in order to cause the release of other proteins and enzymes to continue with the apoptotic process. Their actions are put into motion due to their hydrophobic residues which make them capable of attaching to the membrane of the mitochondria to induce the permeabilization process. Bcl-2 (B-cell lymphoma 2) is an anti-apoptotic protein whereas Bax is a pro-apoptotic protein. There are three domains that this family of proteins contains, BH1, 2, and 3. The Bcl-2 family's mechanism of action is proposed to be the neutralization of pro-apoptotic BH3-only proteins and consequently inhibit their effects on Bax/Bak activity and mitochondrial outer-membrane permeabilization. Conformational changes of Bax and multimer formation with other Bcl-2 family members allow activation and release of other apoptotic proteins from the same family (Tsujimoto, 2003). These proteins act closely together signaling one another in a cascade to promote the apoptosis process (Jourdain and Martinou, 2009, Tsujimoto, 2003).

C. Treatment options

Borek (2004) reports that almost all anti-cancer drugs work by affecting DNA synthesis; they do not kill resting cells unless those cells divide soon after exposure to the drug. Consequently, the efficacy of anti-cancer drugs used in chemotherapy is limited by the fraction of actively dividing cells. Most anti-cancer drugs do not rely on ROS generation for aid in anti-cancer activity, although a few produce free radicals that play a role in treatment such as bleomycin, which produces superoxide radicals. Other anti-cancer drugs include doxorubicin, cisplatin, and bleomycin. Bleomycin is more toxic to oxygenated cells, similar to x-rays and γ -rays, while doxorubicin is preferentially toxic to hypoxic cells (Borek, 2004 A).

The potential effect of antioxidants in enhancing efficacy of chemotherapy treatment depends on the type of drug in use (Borek, 2004 A). Antioxidant protection of normal cells would occur, in principle, in all treatments even when the mechanism is independent of free radical action. Antioxidants help maintain the integrity of normal tissues and protect them from such damaging effects (Borek, 2004 A).

According to Yoon et al., (2007) in cancer treatment, some chemotherapeutic drugs that induce apoptosis lose their pro-apoptotic activities because they activate nuclear factor κ B (NF- κ B), so in order to prevent this from occurring, the inhibition of this transcription factor can restore the mechanisms of action of chemotherapeutic agents to induce apoptosis therefore inhibiting cancer cell viability and proliferation. NF- κ B is a transcription factor that regulates inflammation, immunity, apoptosis, cell proliferation and differentiation. NF- κ B is normally inactive by being bound to an inhibitory protein

(I κ B- α) in the cytoplasm. However, numerous extracellular stimuli including bacteria, viruses, inflammatory cytokines, growth factors, ultraviolet, and oxidative stress cause phosphorylation of the inhibitory protein I κ B- α by I κ B kinase (IKK). This causes the ubiquitination and degradation of I κ B- α by proteasome to release NF- κ B. The released NF- κ B migrates into the nucleus to bind DNA to activate the transcription of inflammatory and other target genes including cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), cyclin D1, and Bcl-2. Activation of NF- κ B induces cancer cell proliferation which brings chemo-resistance to cancer cells (Yoon and Liu, 2007).

Another treatment option in cancer is radiotherapy which uses mostly x-rays and γ -rays and to a lesser extent heavy particle radiation such as protons and neutrons (Borek, 2004 A). Radiation may be classified as directly or indirectly ionizing. When any form of radiation is absorbed in tissues, there is a possibility that it may interact directly with cellular targets. In the direct action, which is dominant when neutrons or protons are considered, the target atoms such as DNA, are directly ionized or excited, initiating events that lead to biological changes. Alternatively, radiation may damage cells by indirect action. In this mode of action, which is dominant in treatment with x-rays and γ -rays, radiation interacts with cellular atoms or molecules to produce free radicals that diffuse far enough to reach and damage critical targets such as DNA. About two thirds of x-ray and γ -ray damage is caused by free radicals that kill tumor cells but threaten the integrity and survival of surrounding normal cells. Radiation induces mitotic cell death in dividing cells and activates pathways that lead to cell death by apoptosis in interphase cells and differentiated cells. The development of cancer produces oxidative stress that increases with disease progression; levels of antioxidants further decrease in response to

treatment, and therapeutic doses of radiation deplete α -tocopherol in normal cells, increasing their risk of damage (Borek, 2004 A).

II. Breast Cancer and the Diet

It has been demonstrated that the diet plays a large role in the development of breast cancer. Some of the studies investigating the relationship of diet and breast cancer are discussed below. A prospective cohort study, also called the Nurses' Health Study II, demonstrated that women with higher red meat consumption during high school had elevated risk of breast cancer whereas adult red meat consumption was not significantly associated with breast cancer (Linos et al., 2008). The researchers also investigated different types of red meat and number of servings per day in relation to breast cancer and found that while greater intakes were associated with increased risk, this was not statistically significant (Linos et al., 2008).

The 4-Corners study, a population-based case-control study of breast cancer, determined that there are no significant or consistent associations in the dietary intake of carotenoids, retinols, vitamin C and tocopherols and breast cancer incidence in Hispanic, American Indian, and non-Hispanic white women aged 25-79 years in Arizona, Colorado, New Mexico, or Utah (Wang, et al., 2008).

The Malmo Diet and Cancer, a population-based cohort of 28,098 individuals living in Malmo, investigated the relationship of diet (particular fiber and energy) and incidence of breast cancer. Sonestedt and colleagues (2008) determined that the mean intake of plant foods did not significantly differ between cases and non-cases of breast tumor incidence. The highest quintile of fiber intake was not significantly associated

with a decrease in the risk of breast cancer whereas the highest quartile of high-fiber bread intake was significantly associated with a 25% decrease in risk when compared to the lowest quintile. Overall, the study concluded that the intake of different plant foods may be differently associated with the risk of breast cancer (Sonestedt et al., 2008).

A case-control study of the relationship of diet and breast cancer risk found a strong protective effect associated with usual intake of vegetables and a less strong association with fruit intake (Freudenheim et al., 1996). Reductions in risk were also associated with vitamin C, α -tocopherol, folic acid, α -carotene, β -carotene, lutein + zeaxanthin, and fiber from vegetables and fruits. The investigators concluded that vegetables appear to exert a protective effect independent of vitamin C, α -tocopherol, folic acid, and fiber (Freudenheim et al., 1996).

In a large population-based case-control study, Malin and colleagues (2003) investigated the intake of fruits, vegetables, and selected micronutrients in relation to the risk of breast cancer and their results suggested that there is no overall association of breast cancer risk with total fruit or vegetable intake. However, there is significant inverse relationship between breast cancer risk and consumption of certain dark green vegetables, dark yellow-orange vegetables, and Chinese white turnips. There was also an inverse relationship observed for all individual fruits except watermelon and apples. Dietary α -tocopherol was also related to a reduced risk of breast cancer (Malin et al., 2003). Fruit and vegetable consumption studies have shown conflicting results for effects on breast cancer risk. This is an area that needs to be further investigated with closer investigation of individual components of fruits and vegetables.

Another study evaluated the dietary intake of Hispanic and a subset of non-Hispanic breast cancer survivors. Hernandez-Valero et al. (2008) found that the overall diets of the two groups were similar and both have an intake of over 5 servings per day of fruits and vegetables. Hispanics consumed significantly more lycopene than the non-Hispanic white women which may be due to cultural differences and incorporation of more tomato products in the Hispanic diet (Hernandez-Valero et al., 2008). The authors also suggested that diet plays a role in the survival of women diagnosed and treated for breast cancer, but needs to be further investigated to determine if higher lycopene intake among the Hispanic survivor participants provides greater protection against breast cancer recurrence or increased survival.

Human observational studies demonstrated that oxidative stress increases with clinical progression of breast cancer and that some dietary antioxidants may have potential benefits in cancer therapy by their ability to induce programmed cell death (apoptosis) (Zimmermann, Bonzon, and Green, 2001). For example, apple extracts have been shown to exert significant inhibitory effects on proteosomal activity of breast cancer cells by inhibition of TNF- α -induced NF- κ B activation (Yoon and Liu, 2007). Vitamins E and C given in combination inhibited apoptosis in endothelial cells by up-regulating the anti-apoptotic protein Bcl-2 and down-regulating the pro-apoptotic Bax (Haendeler, et al., 1996). Studies have shown that vitamin E induced apoptosis in human breast cancer cells (Sigounas, et al., 1997).

In addition to human studies, animal studies have also been conducted to determine the relationship of diet and cancer and to determine the mechanism of action. Moselhy and Al mslmani (2008) investigated the effects of lycopene alone or in

combination with melatonin to determine the inhibition of oxidative stress and carcinogenic effect of DMBA-induced mammary cancer in rats. Protection against tumor-induced carcinogenesis was 66.5% and 80% in the rats receiving lycopene alone and combination of lycopene and melatonin, respectively. Fewest deaths were observed in the lycopene with melatonin group followed by the lycopene group compared to the DMBA group. There were higher antioxidant enzyme activities in the combination treated group followed by the lycopene treated group in comparison to control. The investigators concluded that the combination of certain well known antioxidants may have anti-mammary tumor potential and need to be further investigated (Moselhy and Almslmani, 2008).

Another animal study investigated the effect of a dietary component, lariciresinol, a plant lignan, on mammary cancer in rats and MCF-7 human breast cancer xenografts in athymic mice (Saarinen et al., 2008). The investigators found that nine-week administration of lariciresinol to rats reduced the growth of DMBA-induced mammary gland tumors measured as a cumulative tumor area. The inhibitory effect of lariciresinol was more pronounced in tumors that developed during the treatment period than those established already before the start of the treatment. In the orthotopic MCF-7 xenograft group, intake of lariciresinol containing diet (100 mg/kg) by estradiol-maintained mice for 5 weeks significantly reduced MCF-7 tumor area. Tumor weights were significantly lower in the group administered the lariciresinol diet compared to control mice. The lariciresinol diet increased the number of apoptotic tumor cells according to the TUNEL assay. Tumor vascularization and microvessel densities were also reduced by the lariciresinol diets compared to control. The investigators observed an inhibition in

mammary cancer growth in two different *in vivo* models by lariciresinol suggesting its possible role in the prevention of one of the leading causes of death worldwide (Saarinen et al., 2008).

The human and animal studies discussed above demonstrate the relationship between diet and cancer. Although inconsistencies in findings exist, it has been shown that diet may play a role in the development and progression of breast cancer. Fruits and vegetables have been investigated as well as particular components that are found in these staples of the human diet. It is recommended by several agencies to increase vegetable and fruit intake as dietary strategy to reduce risk of chronic disease and cancer (World Cancer Research Fund/American Institute for Cancer Research, 1997, Van Gils et al., 2005). Further research is warranted so that we may better understand the importance of maintaining a healthy diet to prevent or inhibit further progression of this devastating and fatal disease.

III. Grapes

A. Components of grapes responsible for anti-cancer properties

Plants in their natural environment undergo many stress factors. These factors include drought, salinity, nutritional deficiency, insulation, adverse climate conditions, pollutants, pathogens, insects, and phytophagy. Their survival depends on natural compounds found in the plant that protect it from such stress factors; many of these natural compounds have recently become of particular interest to the nutrition and pharmacological industries due to their disease-counteractive effects (Iriti and Faoro, 2006).

There are many compounds found in grapes that have become of recent interest to researchers in the field of nutrition. Grape components have demonstrated antioxidant, cardioprotective, hepatoprotective, anticarcinogenic, antimicrobial, antiviral, central nervous system, dermatological, and antidiabetic effects (Nassiri-Asl and Hosseinzadeh, 2009). Active constituents of grapes that have been reported are polyphenolic compounds such as flavonoids, anthocyanins, and stilbene derivatives (Nassiri-Asl and Hosseinzadeh, 2009). The amount of these constituents present in grapes depends on several factors including the cultivar, year of production (climate), site of production (geographic origin, soil quality, and fertilization), degree of maturation, and color (Shi et al., 2003).

Polyphenols are found in the skin, pulp, and seeds of the grape; as much as 60 to 70% are in the seeds, very low amounts in the pulp, and as much as 30% in the skin. Flavan derivatives are the common polyphenols and are found as dimers, trimers, and oligomers in the seed and are commonly referred to as procyanidins or proanthocyanidins (Zhao et al., 1999). The amount of these constituents present in grapes depends on several factors including the cultivar, year of production (climate), site of production (geographic origin, soil quality, and fertilization), degree of maturation, and color (Shi et al., 2003).

Flavonoids are antioxidants present in the grape skin and seeds (Yilmaz and Toledo, 2004); these compounds are flavan-3-ols which are colorless and include epicatechin and catechin. The colored compounds are flavanones such as quercetin and red and blue anthocyanins (Shi et al, 2003).

Anthocyanins are responsible for the blue, purple and red color of many plant tissues, including grapes (Prior and Wu, 2006). Common anthocyanin compounds in grapes are glucosides, caffeoylglucosides, diglucosides of cyanidins, delphinidin, peonidin, petunidin, and malvidin (Wang et al., 2003).

The major stilbene derivative found in grapes is resveratrol (Iriti and Faoro, 2006). This compound has become quite commonly used in the research field. There are many active compounds found in grapes which give grapes powerful anti-carcinogenic capabilities.

B. Grapes and breast cancer

Because of the numerous phenolic compounds found in grapes, these compounds have become of particular interest as of late on their role in the prevention of cancer. The next section discusses studies that have been conducted on the relationship of grape intake and breast cancer.

***In vitro* studies**

Singletary et al. (2007) examined the effects of anthocyanin-rich extract from Concord grapes and anthocyanin delphinidin on human breast epithelial cells with DNA damage from an environmental carcinogen, benzo[a]pyrene (BP). Concord grape extract

(CGE) inhibited BP-induced DNA adduct formation in the MCF-10F cell line. These findings indicate that in chemically induced breast carcinogenesis, CGE and select constituent anthocyanins may be considered as potential blocking agents (Singletary et al., 2007).

Sharma and colleagues (2004) assessed the effects of grape seed extract (GSE) and Doxorubicin (Dox), a conventional chemotherapeutic agent, individually or in combination on MCF-7, MDA-MB468, and MDA-MB231 human breast carcinoma cell growth, cell-cycle progression, and apoptosis. GSE and Dox dose- and time-dependently inhibited growth and induced death in these cancer cell lines. Combination of GSE and Dox showed increased therapeutic effect in all the three cell lines. The combination treatment also caused accumulation of MCF-7 cells in G1 phase possibly having an inhibitory effect on the G1-S transition in cell-cycle progression. GSE caused apoptotic death in MCF-7 cells and comparable effect was observed in combination with Dox. MDA-MB468 cells treated with GSE showed strong apoptotic death and the combination of the two treatments caused significant increase in apoptotic death. The authors suggest that a combination of a dietary supplement like GSE along with a reduced dosage of a conventional chemotherapeutic agent such as Dox may enhance its therapeutic effects on breast cancer (Sharma et al., 2004).

Red wine also contains some polyphenols commonly found in red grapes and have been investigated for their role in breast cancer prevention. Red wine polyphenols decrease *in vitro* breast cancer cell proliferation (Damianaki et al., 2000). Hakimuddin and colleagues (2004) examined the anti-proliferative activity of three different structural classes of flavonoids from wine on breast cancer cell lines, MCF-10A and MCF-7, and

one normal human mammary epithelial cell line (HMEC). HMECs were most sensitive to the inhibition of the flavonoids as compared to MCF-10A and MCF-7 cell lines. The less hydrophobic fraction (the crude fraction) of the wine showed similar inhibitory effects on all three cell lines while the more hydrophobic fraction showed the most inhibition of MCF-7 cancer cell line proliferation but not the other two cell lines. The authors suggest that these results may be attributed to flavonoid fractions and growth inhibition is highly dependent on flavonoid concentration. They also suggested that it may be beneficial to increase these components in wine and thus increase the functional food value of the beverage (Hakimuddin et al., 2004).

Another study (Faria et al., 2006) evaluated the effects of procyanidin fractions on MCF-7 human breast carcinoma cells. Catechin fractions (30 and 60 $\mu\text{g/mL}$) caused a decrease in cell viability and inhibition of cell proliferation of MCF-7 cells. The procyanidin fractions that inhibited cell proliferation exhibited higher antioxidant activity. The authors suggest that the antioxidant activity of procyanidins may be partly responsible for the effect on cell proliferation (Faria et al., 2006).

Mantena et al. (2006) examined the effects of grape seed proanthocyanidins (GSPs) in one mouse metastatic breast cancer cell line (4T1) and two human breast cancer cell lines (MCF-7, and MDA-MB-468). 4T1 cells treated with GSPs resulted in a dose-dependent inhibition of cell viability and proliferation as well as induction of apoptosis. Similarly, treatment of the two human cell lines resulted in significant reduction in cell viability and concentration- and time-dependent manner. Bcl-2 expression was decreased and Bax expression increased in 4T1 cells with GSPs treatment, suggesting that this may be responsible for the induction of apoptosis in the

4T1 cells. There was also a dose- and time-dependent increase in levels of cytochrome c Apaf-1 and cleaved caspase-3 with GSPs treatment (Mantena et al., 2006).

Overall, the findings of the *in vitro* studies demonstrate that components in grapes such as polyphenols and flavonoids inhibit cell proliferation and induce apoptosis.

Animal studies

Animal models have also been utilized to investigate the anti-cancer properties of grapes and its components. Mantena et al. (2006) gave immunocompetent Balb/c mice a control diet or diet consisting of 0.2% and 0.5% w/w GSPs. Dietary GSPs significantly inhibited the growth of subcutaneous tumors and increased survival rate in immune competent Balb/c mice inoculated with 4T1 breast cancer cells. GSP also inhibited metastasis of the 4T1 cells from the primary tumor site to the lungs, decreased expression of antiapoptotic Bcl-2, increased Bax, cytochrome c, and Apaf-1 expression as well as activate caspase-3 in the tumors compared to control mice. The authors suggest that the presence of cleaved caspase-3 *in vivo* further confirms that GSPs induce apoptosis in tumor cells through disruption of mitochondrial pathways (Mantena et al., 2006).

Investigators have discovered that red wine polyphenols delay progression of mammary tumors in the transgenic *in vivo* model (Clifford et al., 1996). Eng and colleagues (2003) investigated the relationship between estrogen, wine polyamide extract, and tumor development. Female BALB/c-*nu-nu*, athymic, non-ovariectomized mice were implanted with 5 mg/60 day time-release androstenedione pellets and gavaged with water (CON) or various concentrations of wine polyamide extract for 42 days, and subcutaneously injected with MCF-7aro cells. As the concentrations of wine extract

increased, estradiol and estrone levels decreased in comparison to control although it was not statistically significant (Eng et al., 2003).

In another experiment (Eng et al., 2003), the effect of endogenous estrogens was eliminated by using athymic nude-ovariectomized female mice. These mice showed a significant reduction in tumor size compared with the androstenedione control mice. The 3x concentrate-treated mice had the most dramatic suppression of tumor growth, having no MCF-7aro growth at injection site. There was only a slight reduction in blood estrogen concentrations after treatment with increasing concentrations of wine extract. The authors suggest that based on the results of the study, *in situ*-produced estrogen has a more negative impact on ER-positive breast tumor growth than circulating estrogen. Moreover, the effect of the grape seed extract may result from different phytochemicals other than procyanidin dimers in the extract (Eng et al., 2003).

Chemopreventive efficacy of grape seed extract (GSE) against DMBA-induced mammary tumorigenesis was investigated using Sprague-Dawley rats (Kim et al., 2004). GSE at a 5% dose showed no effect on multiplicity of DMBA-induced mammary tumors; diet showed significant tumor attenuation. Supplementation of 1.25% GSE showed no detectable chemopreventive effect (Kim et al., 2004). This warrants further studies as to the exact mechanism of action of GSE on tumor induction and inhibition in order to know the potential benefits of the extract.

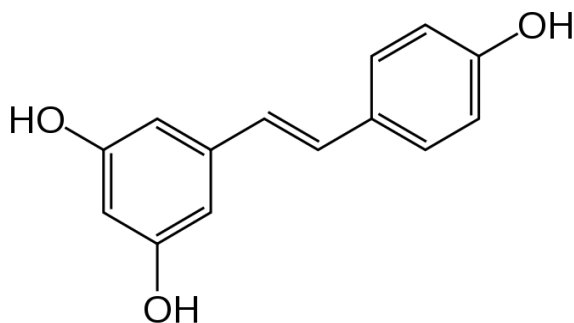
In addition to grape seed extract, purple grape juice has also been used to examine its effects on DMBA-induced rat mammary tumorigenesis and *in vivo* DMBA-DNA adduct formation (Jung et al., 2006). Female Sprague-Dawley rats were provided with drinking water containing diluted Concord grape juice for three weeks prior to DMBA

intubation. There was significant inhibition of *in vivo* mammary DMBA-DNA adduct formation and tumors. During the first 11 weeks tumors were significantly suppressed in rats receiving the grape juice. The constituents or combination of components in the Concord grape juice responsible for the adduct or tumor changes need to be further explored (Jung et al., 2006). There need to be more studies conducted to eliminate the uncertainties related to the active antioxidant components in grapes.

C. Resveratrol and breast cancer

Resveratrol is a naturally occurring phytochemical that is found in approximately 72 plant species, including grapes, peanuts, and various herbs. It is present at particularly high concentrations in grape skin and is also abundant in red wine (Jang et al., 1997). Resveratrol is structurally similar to estrogens which may have implications for the compound as an anticancer agent in breast cancer (**Figure 1**). It has been suggested that it plays an important role in cancer prevention as a therapeutic agent (Jang et al., 1997). Investigators observed that binding of resveratrol to estrogen receptor alpha activates transcription of genes regulated by estrogen (Gehm et al., 1997). Studies have shown that resveratrol inhibits growth of both estrogen receptor alpha positive and negative cell lines (Lu and Serrero, 1999, Mgbonyebi et al., 1998).

Figure 1. Structure of resveratrol.



A few studies have focused on the possible molecular targets of resveratrol to explain its anti-proliferative/apoptotic effects. The induction of cell cycle arrest by resveratrol has been associated to a decreased level of cyclin D1 (Joe et al., 2002), and up-regulation of both the oncosuppressor p53 and the cyclin kinase inhibitor p21/Waf1/CIP1 (Narayanan et al., 2003). Resveratrol was shown to inhibit other known key regulators of cell proliferation such as PKC (Stewart et al., 1999). Induction of cell death by resveratrol was shown to be associated to Bcl-2 phosphorylation (Tinhofer et al., 2001), Bax mitochondrial translocation (Mahyar-Roemer et al., 2002), and inhibition of AKT and FAK activity (Brownson et al., 2002). Resveratrol was also reported to inhibit ribonucleotide reductase, therefore interfering with DNA synthesis in proliferating cancer cells (Fontecave et al., 1998). Moreover, resveratrol was shown to inhibit both the activity and transcription of COX2 (Subbaramaiah et al., 1998).

Some studies show that low concentrations of resveratrol have been found to cause proliferation of ER-positive human breast cancer cells; whereas high concentrations have been found to inhibit the growth of both ER-positive and ER-negative human breast cancer cell lines (Nakagawa et al., 2001). Growth suppression by

resveratrol is the result of mid to late cell cycle arrest (Joe et al., 2002) and modulation of the apoptosis cascade (up-regulation of proapoptotic proteins Bax and Bak, down-regulation of antiapoptotic protein Bcl-x_L, and activation of caspase-3) (Nakagawa et al., 2001).

In vitro studies

The effects of resveratrol on breast cancer using various breast cancer cell lines will be discussed in this section. Exposure of MCF-7 human breast cancer cells to resveratrol (12.5-100 µM) for 48 hours resulted in a dose-dependent cell cycle arrest and apoptosis (Kim et al., 2004). The anti-proliferative effect of the compound was associated with an up-regulation of tumor suppressor p53 and cyclin-dependent kinase (Cdk) inhibitor p21. The increase in apoptosis by resveratrol was connected with an increase in pro-apoptotic protein, Bax, expression, decrease in anti-apoptotic protein, Bcl-2 and Bcl-x_L, expression, and activation of caspase-9. Cyclins and cyclin kinases expressed in the early cell cycle processes showed marked inhibition indicating cell cycle arrest in the G1 and S-phases. Cells treated with high concentrations of resveratrol (50 µM) showed an accumulation of cells in the early phases of the cell cycle as compared to the controls which showed an increased amount of cells in the late and final phases of the cell cycle after 48 hours (Kim et al., 2004).

Laux and colleagues (2004) examined the possible role of p53-dependent pathways in prevention of breast cancer by resveratrol. Five human breast cancer cell lines and a normal cell line, MDA-MB231, MDA-MB 453, MDA-MB 468, BT 20, SKB3, and N132 1N1, respectively were treated with different concentrations of

resveratrol to determine the correlation between p53 and resveratrol-induced apoptosis. Apoptosis was induced in only one cell line, MDA-MB-468, specifically at 10 mM and 40 mM concentrations of resveratrol. The lower concentration of resveratrol showed indications of apoptosis but to a lesser extent. The cell lines that failed to show any changes characteristic of apoptosis have abundant protein levels of p53 (tumor suppressor) or mutant p53 genes and the cell line that showed evidence of apoptosis had low levels of this protein. The investigators suggest that these findings confirm that resveratrol may be used as effective indicator of p53 status of breast cancer cells. Further investigation of the mechanism by which resveratrol induces apoptosis in cells with wild-type p53 is needed. This will further clarify any potential that resveratrol may have in the treatment and prevention of cancer (Laux et al., 2004).

The effect of the (E)- and (Z)-isomers of resveratrol on MCF-7 breast cancer cellular proliferation and the luciferase activity of the MVLN cell line was investigated by Basly and colleagues (2000). Their findings showed that the (Z)-isomer of resveratrol was less effective than the (E)-isomer on the inhibition of cellular proliferation in both MCF-7 and MVLN cell lines. Further research is needed on the effect of the different isomers of resveratrol to determine anticancer properties of each (Basly et al., 2000).

In MCF-7 breast cancer cells, apoptosis was concentration-dependent and found to be highest at a concentration of 150 μ M (Pozo-Guisado et al., 2005). It was also observed that in a dose-dependent manner resveratrol decreased Bcl-2 protein content, an anti-apoptotic protein. This decrease was inversely proportional to the dose of resveratrol. Only very low levels of the protein were observed at 150 μ M, the highest

concentration of resveratrol treatment. Resveratrol was unable to induce caspase-3 and caspase-8 activity, pro-apoptotic enzymes, in the cell line (Pozo-Guisado et al., 2005).

An analogue of resveratrol (3,3',4,4',5,5'-hexahydroxystilbene) was shown to inhibit cell growth in three breast cancer cell lines in a concentration-dependent manner (Murias et al, 2008). In two of the cell lines (ZR-75-1 and T47D) caspase-3 and caspase-9 were detected while all three caspases (caspases -3, -8, and -9) showed activation in MDA-MB-231 cells. The three cell lines exhibited a concentration-dependent loss of mitochondrial potential while 50 μ M of the analogue caused a time-dependent loss. After 24 hours of incubation with 50 μ M of the analogue, cells lines exhibited apoptotic changes and signs of necrosis. There was a time-dependent increase of p53, a tumor suppressor that acts in processes that affect cellular proliferation and survival, in all three cell lines. The highest concentration of p53 was observed after 24 hours of incubation with the resveratrol analogue. The results of this study suggest that apoptosis induced by the resveratrol analogue was mitochondrial in origin as shown by the dissipation of mitochondrial membrane potential that is closely followed by caspase-9 activation (Murias et al, 2008).

Zahid and colleagues (2008) reported that resveratrol dose- and time-dependently induced expression of the phase II enzyme quinone oxidoreductase (NQO1), a protective enzyme, by two- to three-fold in human breast epithelial cells, MCF-10. When cells were preincubated with resveratrol and estrogen-containing medium, NQO1 induction caused a 2-fold decrease in DNA adduct formation at all three doses of estrogen. From these results, the investigators suggest that resveratrol induces the protective enzyme NQO1 by reducing catechol estrogen quinones which react with DNA to form depurinating

adducts that can cause damage to tissues that can possibly lead to carcinogenesis (Zahid et al., 2008).

Animal studies

Resveratrol has been shown to exert anti-carcinogenic activity in animal models of mammary carcinogenesis. Bhat et al. (2001) reported that dietary administration of 10 or 100 mg/kg resveratrol 5 days per week, starting 7 days before administration of a cancer-inducing compound and continuing for the entire period of the study, suppressed induction of mammary carcinomas. At the end of the study (120 days after the administration of the cancer –inducing compound), 100mg/kg resveratrol was found to have reduced the incidence of tumors, decreased the total number of tumors, and increased the latency, compared with untreated controls. Resveratrol at a dose of 10 mg/kg had significantly weaker effects (Bhat et al., 2001).

Resveratrol was found to significantly inhibit tumor growth in female athymic mice injected subcutaneously with MB-MDA-231 human breast cancer cells (Garvin et al., 2006). After three weeks, tumors in the untreated group were five times larger than those of the resveratrol-treated group after only three weeks. Moreover, an increase in apoptosis and decreased angiogenesis was observed in resveratrol-treated tumors compared to control (Garvin et al., 2006).

A study by Whitsett et al. (2006) showed that rats given dietary resveratrol coupled with DMBA to induce carcinogenesis had significantly lower tumor multiplicity and significantly longer tumor latency compared to control. Chemically-induced mammary tumor incidence per rat was lowest in the group receiving resveratrol.

Compared to control animals, resveratrol-exposed rats had significantly delayed onset of first mammary tumor. There was an increase in mammary epithelial cell apoptosis in the terminal end buds and significantly reduced cellular proliferation. The authors suggest that this coupling could create an environment less susceptible to chemical carcinogenesis in the mammary gland (Whitsett et al., 2006).

D. Oklahoma grapes

The Oklahoma grape industry has had a difficult time in the past sustaining the growth and distribution of grapes for product creation and use. Not only does the Oklahoma climate produce some difficulty, but Prohibition laws in the early 1920s dampened the industry (McCraw et al., 2005). Demand for grapes in Oklahoma increased in the 1970s and declined in the 1980s. Of the varieties that have survived the years of difficulty, most contain native *Vitis* parentage which has enabled them to withstand the harsh Oklahoma weather. The efforts of researchers have enabled the release of years of work on desirable fruit characteristics, vine vigor, disease resistance, productivity, drought resistance, cold hardiness, and fruit acceptability for juice, jelly, table use, and wine production.

Since the Oklahoma grape industry has suffered hardships, commercial grape production is relatively new to many areas of Oklahoma. There are many things to consider when planning a vineyard, including microclimate, soil, irrigation, and disease and pest management for example (McCraw et al., 2005). There has been a steady increase in grape production since the mid-1990s. There has also been a recent increase in Oklahoma vineyards and grape production; 375 acres of grapes were grown in 2002

and increased to 525 acres in 2005. Oklahoma wineries are also on the rise; there were four licensed wineries in 2001 and as of 2007, there are 50. These numbers show the expansion of the Oklahoma grape and wine industry in a short period of time and the importance of the research industry in producing the disease resistant, cold hardy varieties. Of the total acreage in Oklahoma used for grape production, 60 percent is for the production of red grapes. Lincoln County reports the largest acreage and more than 10 vineyards. There are more than 40 grape varieties currently being produced in Oklahoma (Stafne, 2005).

The Oklahoma grape industry has greatly expanded in the past 50 years. Many researchers have added to the growth of the industry through efforts to create and produce types of grapes able to resist disease and Oklahoma weather changes. Since the state's industry is still relatively new and expanding, the research being conducted using Oklahoma grape varieties to assess their potential in disease prevention and treatment is new. This is one of the reasons why we chose an Oklahoma grape variety, Rubaiyat, to assess its role in preventing breast cancer with widely used MCF-7 human breast cancer cell line.

E. Rubaiyat grapes

It has been demonstrated that phenolic compound found in grapes play a role in the prevention of breast cancer. As mentioned earlier, phenolic content of grapes can be influenced by several factors such as the growing environment and the variety of grapes. To our knowledge, this is the first study investigating the cancer preventing properties of Rubaiyat grapes. We have chosen this particular grape variety due to its relatively high

total phenolic content. Our study examines the effect of the phenolic-rich extract of Rubaiyat grapes, a native Oklahoma grape, in inhibiting cellular proliferation and inducing apoptosis using a commonly used breast cancer cell line, human MCF-7.

The Rubaiyat grape is a cross between Seibel 5437 and 'Bailey' made at Oklahoma State University developed in 1952 by Herman Hinrichs. Seibel is a black grape that ripened early to midseason, cold hardy, and has medium size clusters and berries (Stafne, 2006). The Rubaiyat seed was first germinated in 1953 after which it was grown in a nursery for two years. It was placed into the field at the Pecan and Fruit Experiment Station (Perkins, OK) in 1955, and fruit was first observed in 1957 (Stafne, 2006). Rubaiyat is a dark blue-black grape variety with medium-sized clusters and berries that ripen in mid-August. The variety has good to very good disease resistance. It produces a very dark red juice which contains 19% sugars and tartaric acid levels of 0.63%. (Stafne, 2006).

CHAPTER III

METHODOLOGY

Preparation of Rubaiyat grape extract

Oklahoma grapes of the Rubaiyat variety were obtained from Perkins Research Station (Perkins, OK). 100 g fresh grapes were cleaned and dried before extraction of phenolics using a method previously reported (Sun and Liu, 2006). Grapes were pureed with a solution of methanol, ethanol, and water (50:25:25, v:v:v). The puree was sonicated in ice for 30 minutes and centrifuged for 30 minutes at 10,000 g at 4°C. The puree was then filtered under vacuum through a #2 Whatman filter paper on a Buchner funnel. The filtered extract was roto-evaporated until no more than approximately 10 mL was remaining. The concentrated extract was run through a C¹⁸ column (Waters, Taunton, Massachusetts) that was pre-conditioned with methanol and Millipore water to obtain polyphenols and discard proteins and sugars. Polyphenols were eluted from the column with methanol. The extract was roto-evaporated once more to evaporate the methanol. The remaining extract was dissolved in water and aliquoted into cryovial tubes in 1.0 mL quantity and stored at -80°C until needed.

Determination of total phenolic content of the grape extract

The total phenolic content of the grape extract was analyzed by the Folin-Ciocalteu colorimetric method described previously (Sun and Liu, 2006). Briefly, gallic acid (Sigma-Aldrich Chemical Co., St. Louis, MO) dissolved in 40% ethanol was used as the standard (0-1000 μ M). Appropriate dilutions of grape extract and standards were oxidized with Folin-Ciocalteu reagent (Sigma-Aldrich Chemical Co., St. Louis, MO) and the reaction was neutralized with 20% sodium carbonate (Fisher Scientific, Fair Lawn, NJ), followed by the addition of Millipore water. After 90 minutes, the absorbance was measured against acidified distilled water as the blank at 760 nm using a BioTek Synergy HT microplate reader (BioTek Instruments Inc., Winooski, VT). Analyses were done in triplicate and the results were expressed as mean μ g/mL.

Determination of total flavonoid content of the grape extract

Total flavonoid content of the grape extract was determined using a colorimetric method described previously (Sun and Liu, 2006). Briefly, 1 mL of grape extract and equivalent solution of catechin (Sigma-Aldrich Chemical Co., St. Louis, MO) in water as standard (0-200 mg/L) was mixed with distilled water followed by addition of 5% sodium nitrite solution (Sigma-Aldrich Chemical Co., St. Louis, MO). After 5 minutes, 10% aluminum chloride solution (Fisher Scientific, Fair Lawn, NJ) was added and allowed to stand for 6 minutes followed by addition of 1 M sodium hydroxide (Sigma-Aldrich Chemical Co., St. Louis, MO). Total volume of the mixture was adjusted with distilled water and the absorbance was immediately measured at 510 nm using the BioTek Synergy HT microplate reader (BioTek Instruments Inc., Winooski, VT). An

equivalent dilution of acidified distilled water was used as blank. Analyses were done in triplicate and the results were expressed as mean $\mu\text{g/mL}$.

Determination of anthocyanin content of the grape extract

Anthocyanin content of the grape extract was determined using a method previously described (Sun and Liu, 2006). Grape extract was diluted with two different buffers separately, one with potassium chloride buffer (0.025 M, pH 1.0) (Sigma-Aldrich Chemical Co., St. Louis, MO) and the other with sodium acetate buffer (0.4 M, pH 4.5) (Sigma-Aldrich Chemical Co., St. Louis, MO). Grape extract was diluted with buffers in a 1:9 ratio of extract to buffer in a 96-well plate and equilibrated for 15 minutes in the dark. The absorbance of each dilution was read at 510 nm and 700 nm using a Biotek Synergy HT microplate reader (Biotek Instruments Inc., Winooski, VT) and measured against a blank cell filled with equivalent dilution of acidified distilled water. Total monomeric anthocyanins content was calculated according to the equation.

$$\text{Anthocyanin concentration} = \Delta A \times \text{MW} \times 1000 / (\epsilon \times C)$$

A, absorbance $[(A_{510} - A_{700}) \text{ pH } 1.0 - (A_{510} - A_{700}) \text{ pH } 4.5 = \Delta A]$; C, grapes concentration (9/1); MW = 449.2; and $\epsilon = 26,900$

Analyses were done in triplicate and the results were expressed as a mean $\mu\text{g/mL}$.

Cell culture and resveratrol

The human breast cancer cell line MCF-7, an ER⁺ epithelial cell line, was obtained from American Type Culture Collection (ATCC, Rockville, MD) and cultured in a T-75 flask containing 25 mL Eagles minimum essential medium (ATCC, Rockville,

MD) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich Chemical Co., St. Louis, MO), 1% penicillin-streptomycin (Sigma-Aldrich Chemical Co., St. Louis, MO), and 10 mg/mL bovine insulin (Sigma-Aldrich Chemical Co., St. Louis, MO) and maintained in a 37°C incubator with 5% CO₂. Resveratrol was purchased from Sigma-Aldrich Chemical Co., (St. Louis, MO, USA) and dissolved in DMSO (Fisher Scientific, Fair Lawn, NJ) as a stock solution at 200 mM concentration, and stored in aliquots at room temperature.

Measurement of inhibition activity towards MCF-7 cell proliferation by sulforhodamine B assay

The cytotoxic effects of grape extract and resveratrol were measured by the sulforhodamine B (SRB) assay (Houghton et al., 2007). Cells were maintained at 37°C with 5% CO₂ in minimum essential medium alpha medium (MEM) containing 10 mg/mL bovine insulin, 50 units/mL penicillin, 50 units/mL streptomycin, and 10% fetal bovine serum. MCF-7 cells in growth media were placed in each well of a 96-well flat-bottom plate at a concentration of 5×10^3 cells/well. After 24 hours of incubation at 37°C with 5% CO₂, the growth medium was replaced by media containing different concentrations of Rubaiyat grape extract (11.25, 22.5, 45, and 228 µg/L) or resveratrol (50, 100, 200, and 1000 µM which is equivalent to 11.25, 22.5, 45, and 228 µg/L of Rubaiyat grape extract, respectively). Control group received growth medium or growth medium with 0.1% DMSO. After 48 or 72 hours of incubation with Rubaiyat grape extract or resveratrol, cells were fixed by the addition of 200 µL of 40% ice-cold trichloroacetic acid (Sigma-Aldrich, St Louis, MO) for one hour at 4°C. Cells were then washed five

times with distilled water and air-dried. Sulphorodamine B solution (Sigma-Aldrich, St. Louis, MO) in 1% acetic acid (0.4% w/v) was added to each well and allowed to stain for 30 minutes at room temperature. SRB solution was removed and the plate was washed five times with 1% (v/v) acetic acid to remove unbound dye and air-dried. The bound SRB was solubilized by adding 100 μ L of 10 mM unbuffered tris base (pH 10.5) to each well and shaken for 5 minutes on a shaker platform. Cytotoxicity of grape extract and resveratrol was determined at 48 and 72 hours from the SRB optical density at 492 nm reading from a Biotek Synergy microplate reader (Biotek Instruments Inc., Winooski, VT) Cell proliferation was expressed as % of control samples. Experiment was repeated three times in triplicate to determine the cytotoxic activity.

Nuclear staining with 4'6-diamidino-2-phenylindole (DAPI)

The apoptotic effects of the Rubaiyat grape extract and resveratrol were measured by the DAPI assay (Lewis-Wambi et al., 2009). Cells were maintained at 37°C with 5% CO₂ in minimum essential medium alpha medium (MEM) containing 10 mg/mL bovine insulin, 50 units/mL penicillin, 50 units/mL streptomycin, and 10% fetal bovine serum. MCF-7 cells in growth media were seeded in a 6-well plate at a concentration of 2.5×10^5 cells/well. After 24 hours of incubation at 37°C with 5% CO₂, the growth medium was replaced by media containing different concentrations of grape extract (11.25, 22.5, 45, and 228 μ g/L) or resveratrol (50, 100, 200, and 1000 μ M which is equivalent to 11.25, 22.5, 45, and 228 μ g/L, respectively). Control group received of growth medium or growth medium with 0.1% DMSO. After 48 or 72 hours of incubation, old media was taken off and discarded and cells were washed three times with PBS, and then

resuspended for 20 minutes in 4% PBS-buffered paraformaldehyde solution containing 10 µg/mL DAPI (Sigma-Aldrich, St. Louis, MO). Cells were then washed with PBS two times and observed using a fluorescence microscope (Nikon Eclipse TE2000-U, Japan) at excitation wavelength of 350 nm. Apoptosis was visualized with the naked eye. DAPI is a blue fluorescent nucleic acid stain that preferentially stains double-stranded DNA by attaching to AT clusters in the DNA minor groove. One molecule of dye binds for each 3 base-pairs (Kapuscinski and Szer, 1979).

Expression of apoptotic genes, Bax and Bcl-2, by real-time RT-PCR

Apoptosis induction was assessed in MCF-7 cells using real time RT-PCR to examine Bax and Bcl-2 gene expression. Cells were maintained at 37°C with 5% CO₂ in minimum essential medium alpha medium (MEM) containing 10 mg/mL bovine insulin, 50 units/mL penicillin, 50 units/mL streptomycin, and 10% fetal bovine serum. MCF-7 cells in growth media were seeded in a 6-well plate at a concentration of 2.0×10^6 cells/well. After 24 h of incubation at 37°C with 5% CO₂, the growth medium was replaced by media containing different concentrations of grape extract (11.25, 22.5, and 45 µg/mL) or resveratrol (50, 100, and 200 µM which is equivalent to 11.25, 22.5, and 45 µg/mL, respectively). Control group received growth medium or growth medium with 0.1% DMSO.

After 48 hours of incubation, media was taken off and RNA extracted with 800 µL STAT-60 solution (Tel-Test Inc., TX). Samples were gently mixed and transferred into RNase-free microfuge tubes and incubated at room temperature in order to promote complete dissociation of RNA-protein complexes. Chloroform (Sigma-Aldrich, St.

Louis, MO) was added to each sample, shaken vigorously, incubated at room temperature, and centrifuged at 12,000 x g at 4 °C for 15 minutes. The aqueous phase containing RNA was transferred to a new RNase-free centrifuge tube. Isopropanol (Sigma-Aldrich, St. Louis, MO) was added to each sample, vortexed, centrifuged at 12,000 x g at 4°C for 10 minutes, and the RNA pellet was saved. RNA pellet was rinsed with 70% ethanol and air-dried for 3-5 minutes. RNA was reconstituted with 30 µL DEPC-H₂O, and the concentration of the RNA was determined on a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). Samples were stored at -80°C until further analyses.

For complementary DNA synthesis, DNase mix (Roche, Indianapolis, IN), 10x PCR buffer (Roche, Indianapolis, IN), DEPC-H₂O and 25 mM MgCl₂ (Sigma-Aldrich, St. Louis, MO) were mixed to get a DNase master mix. The master mix was added to each RNA sample and placed in a thermocycler to incubate at 37°C for 30 min. The enzyme present in the samples was then heat inactivated at 75°C for 10 min. A reverse transcription was performed by mixing 5x buffer (Invitrogen, Carlsbad, CA), 0.1 M DTT (Invitrogen, Carlsbad, CA), dNTP mix (Promega, Madison, WI), 0.8 mg/mL pdN6 (Roche), DEPC-H₂O, and RTase (Invitrogen) to each sample. Samples were incubated in a thermocycler at 25°C for 10 min, 42°C for 50 min, and 72°C for 10 min, then a 4°C soak cycle.

Each real-time reaction was performed using SYBR Green chemistry (Applied Biosystems, Foster City, CA). Primer Express Software (Perkin-Elmer Life Sciences) was used to design primers for each gene (Valasek et al., 2007). Primers were validated by analysis of template titration and dissociation curves (Valasek et al., 2007).

Genes involved in cellular apoptosis induction, Bax and Bcl-2 (pro- and anti-apoptotic proteins) were assessed. Cyclophilin was the housekeeping gene. Primer sequences as follows: hBAX-QF1-5'TGGAGCTGCAGAGGATGATTG, hBAX-QR1-5'GCTGCCACTCGGAAAAAGAC, hBCL2-QF1- 5'GGGATGCCTTTGTGGAAGT, hBCL2-QR1- 5'CAGCCAGGAGAAATCAAACAGA, hCyclo-QF- 5'TGCCATCGCCAAGGAGTAG, hCyclo-QR- 5'TGCACAGACGGTCACTCAAA. Amplification of RNA was detected constantly by real-time quantitative PCR using an ABI Fast 7900HT Real-Time PCR System (Applied Biosystems, Foster City, CA). Cycles included one 2 minute hold (50°C); one 10 minute denaturation (95°C); 40 cycles of denaturation (95°C for 15 seconds). All real-time PCR results were analyzed using the comparative cycle number at threshold (C_T) method.

Statistical analyses

Statistical analyses involved computation of least square means and standard deviation (SD) for each of the treatment groups. Analyses of variance (ANOVA) was conducted using SAS version 9.1 (SAS Institute, Cary, NC) with PROC GLM to determine significance of treatment, concentration, and duration of treatment effects. If post hoc analysis shows statistical significance, Fisher's least square means separation test was used to determine and compare the significant differences among the means. Differences were considered significant at $P < 0.05$.

CHAPTER IV

RESULTS

Total phenolic, flavonoid, and anthocyanin content of the grape extract

Total phenolic content of the Rubaiyat grape extract used in the study as determined by the Folin-Ciocalteu method was found to be approximately 1140 µg/mL (**Table 1**). Anthocyanin and flavonoid of the grape extract was determined to be approximately 66 and 620 µg/mL, respectively (**Table 1**).

Cell proliferation by sulforhodamine B assay

The sulforhodamine B assay was used to measure the cytotoxicity on human MCF-7 breast cancer cells of various concentrations of treatment with Rubaiyat grape extract or resveratrol after 48 and 72 hours. Overall, there was no significant difference in treatment duration (48 vs 72 hours) (**Table 2**). However, looking at the individual treatment, cellular proliferation was significantly reduced by resveratrol after 72 hours compared to treatment duration of 48 hours (**Figure 2**). This reduction in cellular proliferation was not observed with the Rubaiyat grape extract ($P= 0.1402$) (**Figure 2**).

Looking at the effect of concentration (regardless of treatment), there was a significant difference in cellular proliferation with different concentrations (**Table 2**). Cellular proliferation was significantly reduced starting at 22.5 $\mu\text{g/mL}$ and lowest at 228 $\mu\text{g/mL}$ (**Table 2**). There was statistical significance when comparing concentration with treatment with time ($P = 0.0078$) (**Table 2**). Looking at resveratrol alone, cellular proliferation was significantly reduced starting at 11.25 $\mu\text{g/mL}$ (50 μM) and the two highest concentrations (45 and 228 $\mu\text{g/mL}$, equivalent to 200 and 1000 μM , respectively) had the highest inhibition of cellular proliferation after treatment for 48 hours; although the two highest concentrations were not statistically different from each other (**Figure 3**). A similar pattern was observed for 72 hours of treatment with resveratrol (**Figure 3**).

It takes more Rubaiyat grape extract to inhibit cellular proliferation of MCF-7 breast cancer cells in comparison to resveratrol (**Figure 4**). Rubaiyat grape extract at 228 $\mu\text{g/mL}$ is the only concentration that inhibited MCF-7 cellular proliferation at 48 and 72 hours of treatment (**Figure 4**). It is interesting to note that Rubaiyat grape extract at 11.25 $\mu\text{g/mL}$ has the highest MCF-7 cell proliferation (**Figure 4**).

Nuclear staining with DAPI

The apoptotic effects of resveratrol and grape extract after 48 and 72 hours of treatment were measured by the 4',6-diamidino-2-phenylindole (DAPI) assay (**Figures 5 and 6**). Treatment for 48 hours with Rubaiyat grape extract at 228 $\mu\text{g/mL}$ showed the most marked induction of apoptosis on human MCF-7 breast cancer cells compared to the control (**Figure 5**). Cells were far less numerous and misshapen, possibly indicating membrane blebbing, and smaller in size, all common apoptosis characteristics. These

characteristics were observed in a dose-dependent manner (**Figure 5**). Resveratrol-treated cells showed these same characteristics in a dose-dependent manner and were observed starting at the lowest concentration ($11.25\text{ }\mu\text{g/mL} = 50\text{ }\mu\text{M}$) and were most marked at $228\text{ }\mu\text{g/mL}$ concentration ($1000\text{ }\mu\text{M}$) (**Figure 5**). Grape extract and resveratrol treatment for 48 hours showed similar results at their highest treatment concentrations ($228\text{ }\mu\text{g/mL} = 1000\text{ }\mu\text{M}$) (**Figure 5**).

At 72 hours of treatment with Rubaiyat grape extract, $228\text{ }\mu\text{g/mL}$ concentration showed greatest apoptosis induction (**Figure 6**). Grape extract treatment showed dose-dependent apoptotic induction (**Figure 6**). Cells showed characteristics of apoptosis, such as membrane blebbing, reduction in cell size, and were less numerous. Resveratrol treatment showed characteristics of apoptosis induction at the lowest concentration ($11.25\text{ }\mu\text{g/mL} = 50\text{ }\mu\text{M}$) and greatest apoptosis induction at $228\text{ }\mu\text{g/mL}$ ($1000\text{ }\mu\text{M}$) after 72 hours (**Figure 6**). It exhibited dose-dependent apoptosis induction and similar apoptosis characteristics. Grape extract treatment at $228\text{ }\mu\text{g/mL}$ concentration exhibited less prominent apoptotic characteristics than resveratrol at $228\text{ }\mu\text{g/mL}$ ($1000\text{ }\mu\text{M}$) (**Figure 6**). Apoptosis induction by both resveratrol and grape extract was both dose- and time-dependent with the highest concentration of each treatment showing most prominent apoptotic characteristics (**Figure 6**). However, all these data analyses need to be interpreted with caution as apoptosis visualization was done using the naked eye and not by a quantitative method.

Expression of apoptotic genes, Bax and Bcl-2, by real-time RT-PCR

The expression of pro- and anti-apoptotic markers was determined by quantitative real-time polymerase chain reaction. There was no significant difference between resveratrol and Rubaiyat grape extract in the relative mRNA expression of the Bax gene (**Table 3**). Moreover, there was no significant difference in the relative mRNA expression of Bax with different concentrations of both resveratrol (**Figure 7 and Table 3**) and Rubaiyat grape extract (**Figure 8 and Table 3**).

The relative mRNA expression for Bcl-2 tended ($P=0.0580$) to be lower with Rubaiyat grape extract compared to resveratrol (**Table 3**). Moreover, there was a significant difference in the relative mRNA expression of this gene with different concentrations of Rubaiyat grape extract and resveratrol (**Table 3**). Treatment with 45 $\mu\text{g/mL}$ (200 μM) of resveratrol significantly down-regulated gene expression of Bcl-2 (**Figure 7**). Lower concentrations of resveratrol (11.25 and 22.5 $\mu\text{g/mL}$ which is equivalent to 50 and 1000 μM , respectively) did not reduce Bcl-2 gene expression (**Figure 7**). However, Rubaiyat grape extract was able to significantly reduce gene expression of Bcl-2 starting at 11.25 $\mu\text{g/mL}$ (**Figure 8**).

Table 1. Total phenolic, anthocyanin, and flavonoid content of the Rubaiyat grape extract used in the study

Parameter ¹	Amount (µg/mL) ²
Total phenolic	1140.5 ± 45.0
Anthocyanin	65.6 ± 17.7
Flavonoid	619.5 ± 40.6

¹Total phenolic, anthocyanin, and flavonoid content were determined by Folin-Ciocalteu, gallic acid colorimetric, and catechin colorimetric methods, respectively.

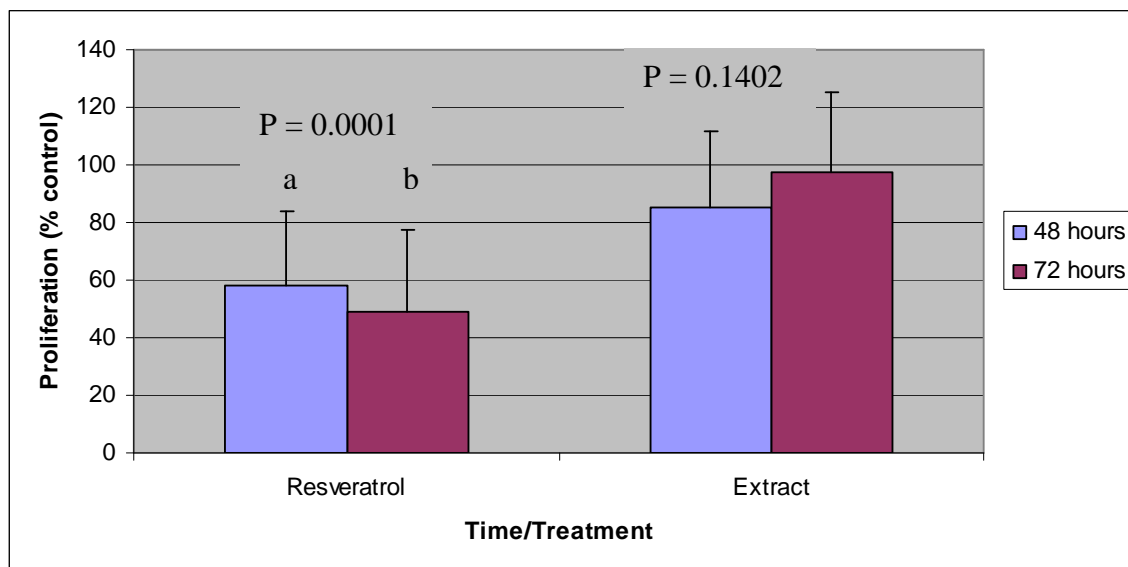
² Values are mean ± SD.

Table 2. Effect of resveratrol and Rubaiyat grape extract treatment for 48 and 72 hours on MCF-7 human breast cancer cell proliferation ¹

Time effect (hours)	Proliferation (% control)
48	71.5±29.2
72	73.2±37.1
Concentration effect (µg/mL)	
0	100.0±0.0 ^a
11.25	86.4±35.4 ^a
22.5	71.4±34.4 ^b
45	58.7±32.4 ^{bc}
228	45.3±18.5 ^c
Treatment effect	
Resveratrol	53.4±27.1 ^B
Rubaiyat grape extract	91.3±27.4 ^A
P values	
Concentration	<0.0001
Treatment	<0.0001
Time	0.6787
Concentration*Treatment*Time	0.0078

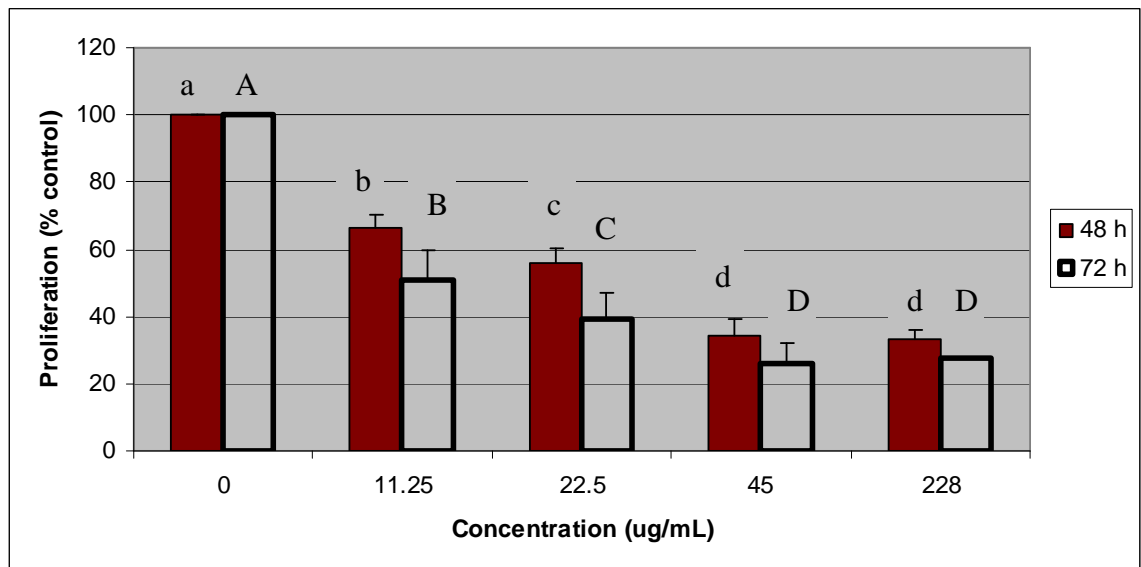
¹Cell proliferation was assessed by the sulforhodamine B assay. Values are mean ± SD, experiment was repeated 3 times. Values that do not share the same letters were significantly different from each other.

Figure 2. Effect of treatment duration with resveratrol and Rubaiyat grape extract on MCF-7 cellular proliferation ¹



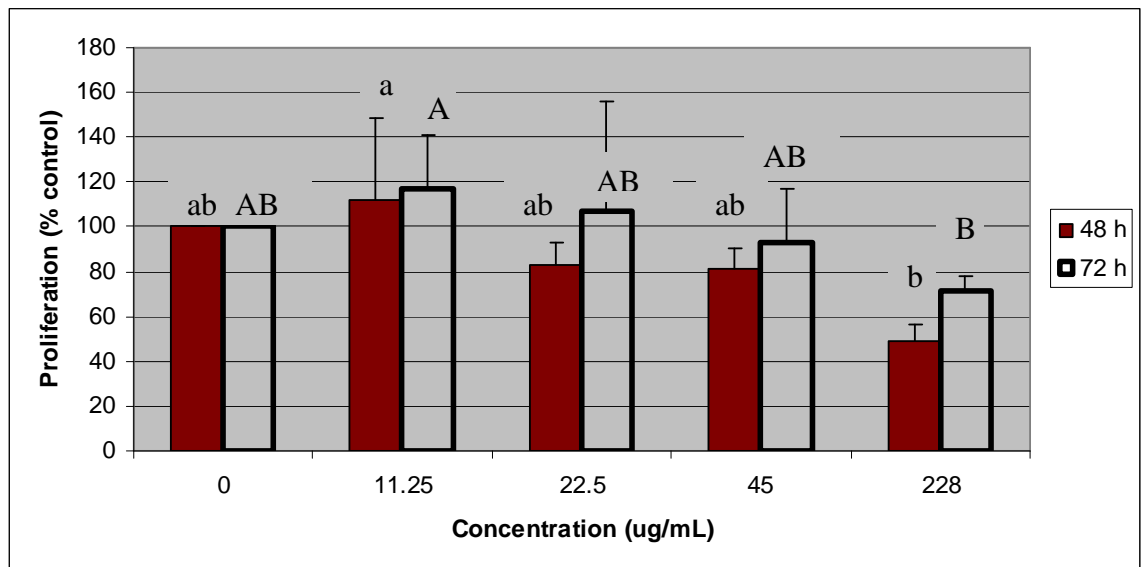
¹ Cell proliferation was assessed by the sulforhodamine B assay. Values are mean \pm SD, experiment was repeated 3 times. Bars that do not share the same letters are significantly ($P < 0.05$) different from each other.

Figure 3. Effect of treatment with different concentrations of resveratrol for 48 or 72 hours on MCF-7 cellular proliferation ¹



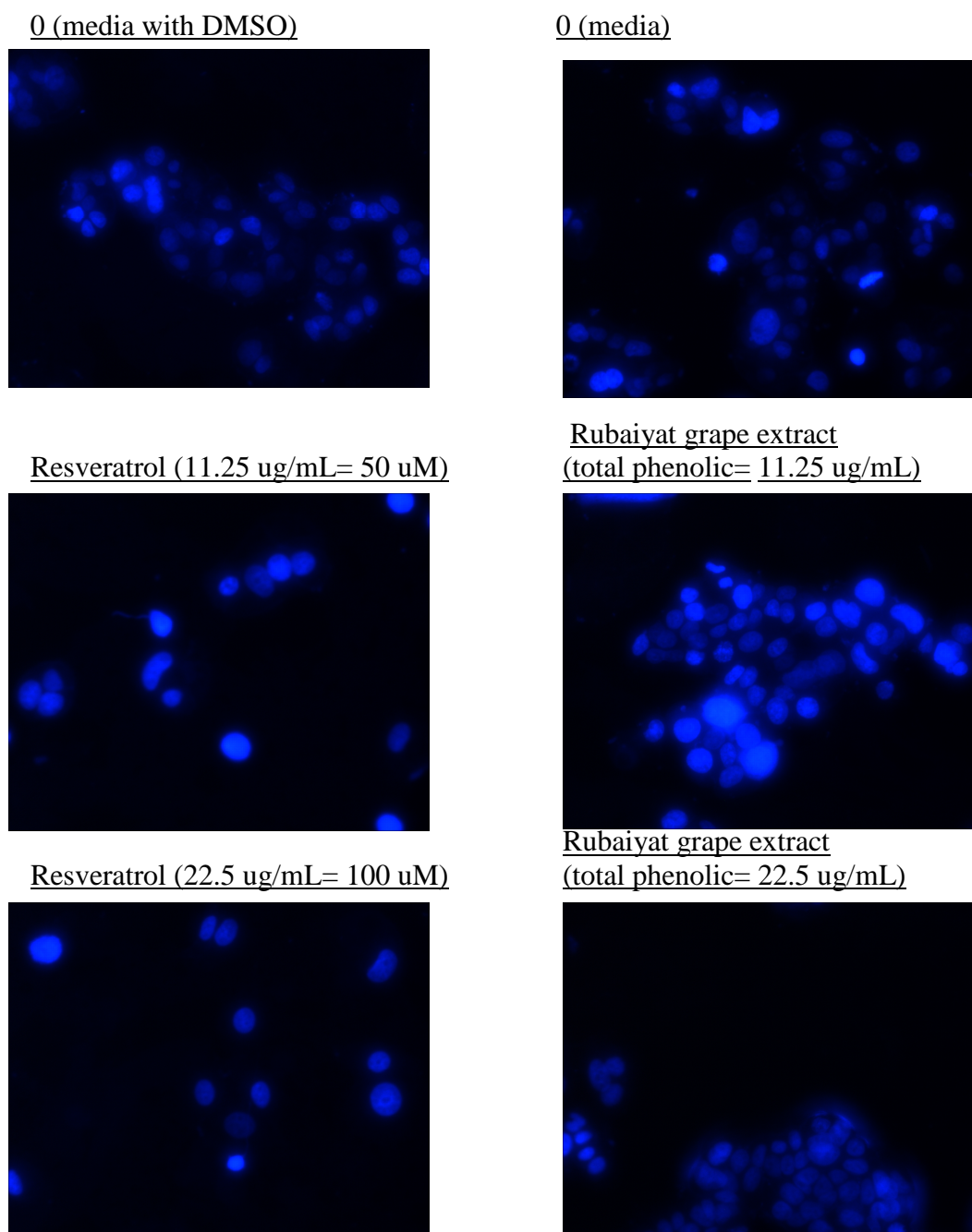
¹ Cell proliferation was assessed by the sulforhodamine B assay. Values are mean \pm SD, experiment was repeated 3 times. Bars that do not share the same letters are significantly ($P < 0.05$) different from each other. Small letters are comparison of different concentrations at 48 hours (48 h) of treatment while capital letters are for 72 hours (72 h).

Figure 4. Effect of treatment with different concentrations of Rubaiyat grape extract for 48 and 72 hours on MCF-7 cellular proliferation ¹

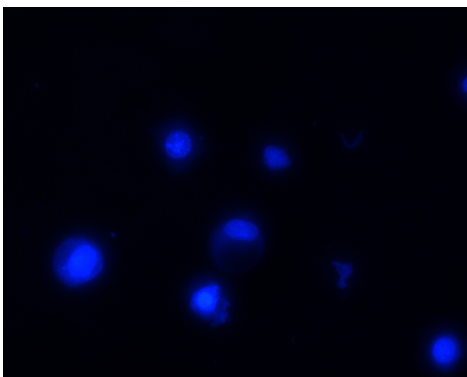


¹ Cell proliferation was assessed by the sulforhodamine B assay. Values are mean \pm SD, experiment was repeated 3 times. Bars that do not share the same letters are significantly ($P < 0.05$) different from each other. Small letters are comparison of different concentrations at 48 hours (48 h) of treatment while capital letters are for 72 hours (72 h).

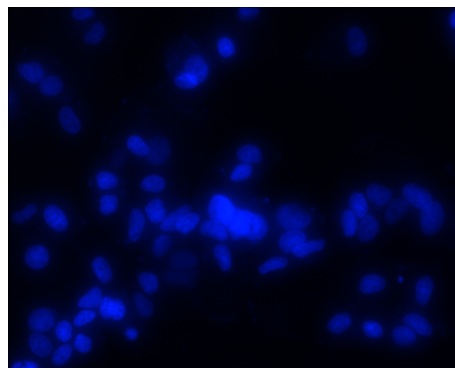
Figure 5. Representative picture of the effect of treatment with different concentrations of resveratrol and Rubaiyat grape extract for 48 hours on MCF-7 human breast cancer cellular apoptosis as assessed by the 4'6-diamidino-2-phenylindole (DAPI) assay



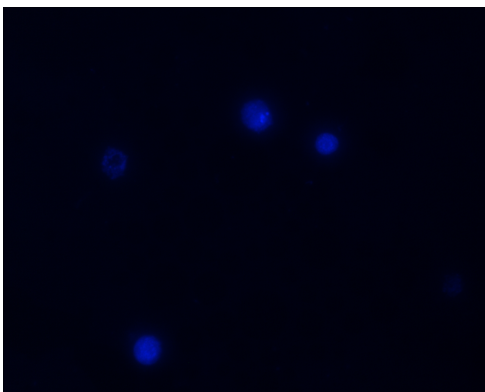
Resveratrol (45 ug/mL= 200 μ M)



Rubaiyat grape extract
(total phenolic= 45 ug/mL)



Resveratrol (228 ug/mL= 1000 μ M)



Rubaiyat grape extract
(total phenolic= 228 ug/mL)

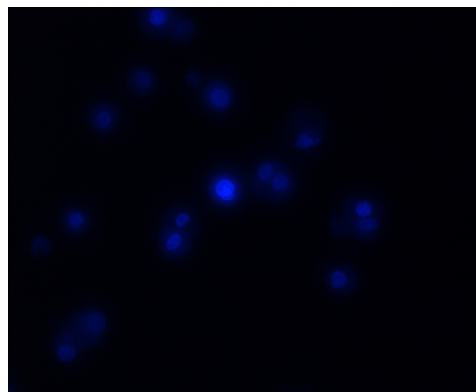
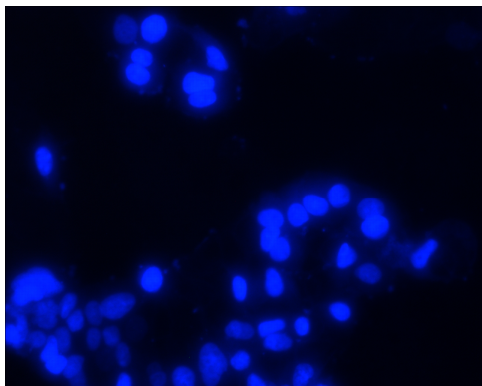
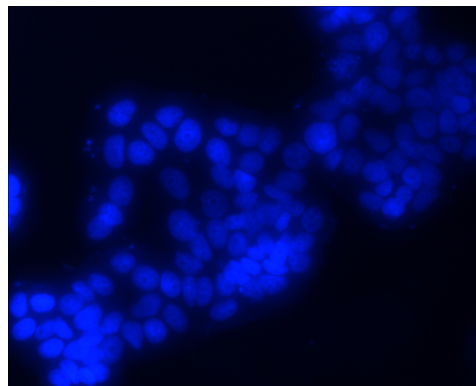


Figure 6. Representative picture of the effect of treatment with different concentrations of resveratrol and Rubaiyat grape extract for 72 hours on MCF-7 human breast cancer cellular apoptosis as assessed by the 4'6-diamidino-2-phenylindole (DAPI) assay

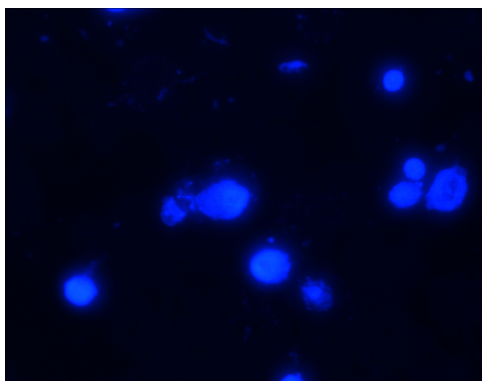
0 (media with DMSO)



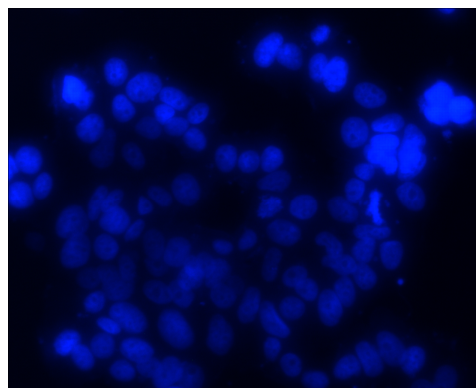
0 (media)



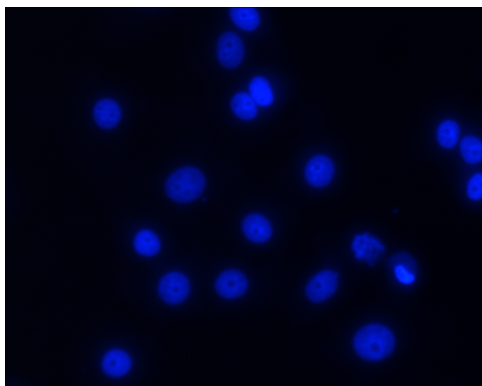
Resveratrol (11.25 ug/mL= 50 μ M)



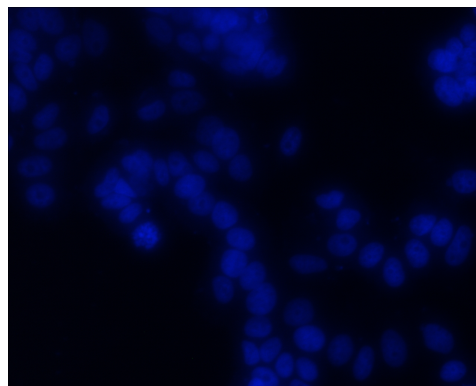
Rubaiyat grape extract
(total phenol= 11.25 μ g/mL)



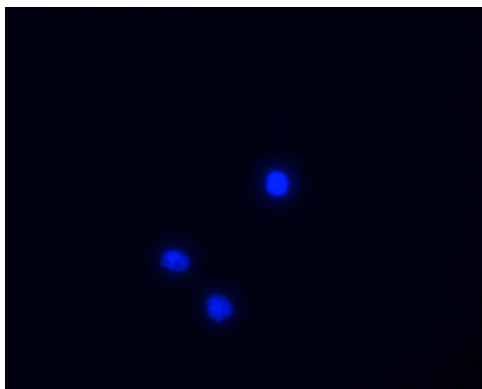
Resveratrol (22.5 ug/mL= 100 μ M)



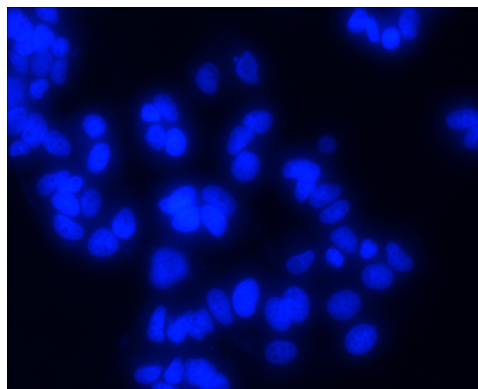
Rubaiyat grape extract
(total phenolic= 22.5 μ g/mL)



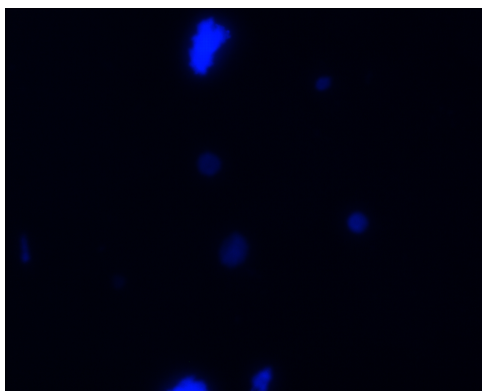
Resveratrol (45 ug/mL= 200 μ M)



Rubaiyat grape extract
(total phenolic= 45 μ g/mL)



Resveratrol (228 ug/mL= 1000 μ M)



Rubaiyat grape extract
(total phenolic= 228 μ g/mL)

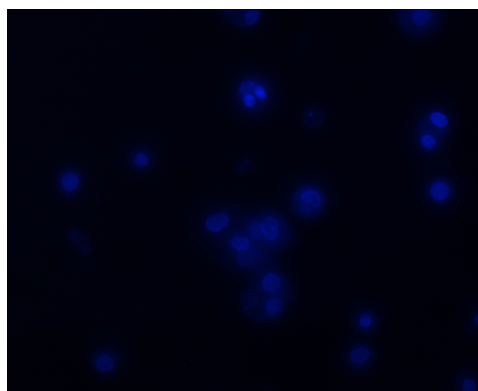
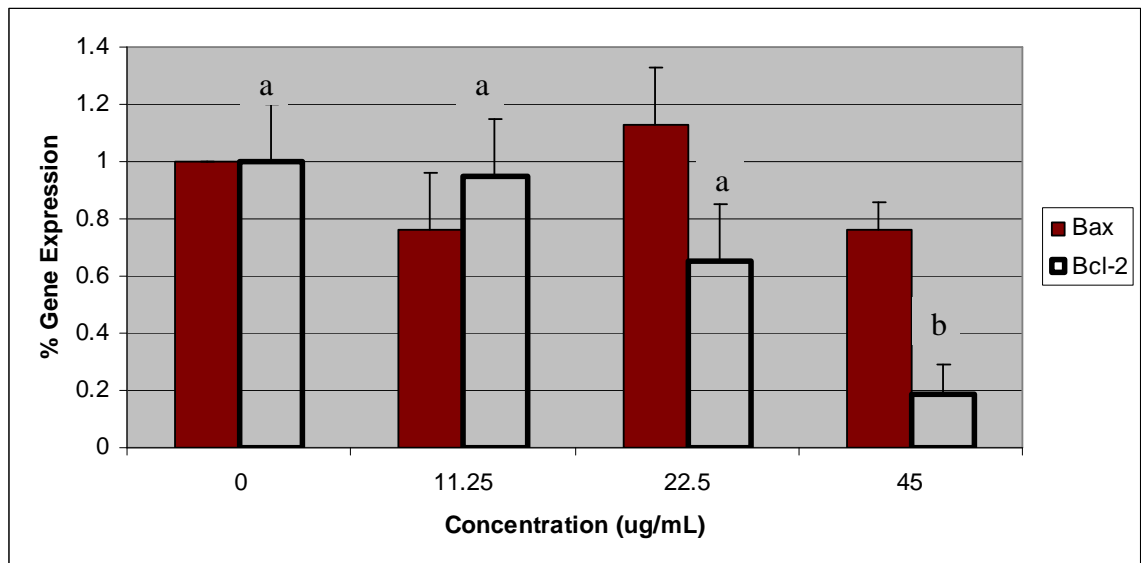


Table 3. Effect of resveratrol and Rubaiyat grape extract treatment for 48 hours on apoptotic gene expression (Bax and Bcl-2) ¹

Treatment effect	Bax relative mRNA (pro-apoptotic)	Bcl-2 relative mRNA (anti-apoptotic)
Resveratrol	0.91±0.22	0.70±0.37
Rubaiyat grape extract	1.01±0.18	0.51±0.38
Concentration effect (µg/mL)		
0	1.00±0.12	1.00±0.12 ^a
11.25	0.92±0.24	0.72±0.39 ^b
22.5	1.02±0.19	0.48±0.23 ^b
45	0.90±0.26	0.20±0.04 ^c
P values		
Concentration	0.5423	0.0002
Treatment	0.2348	0.0580
Treatment*concentration	0.1012	0.1747

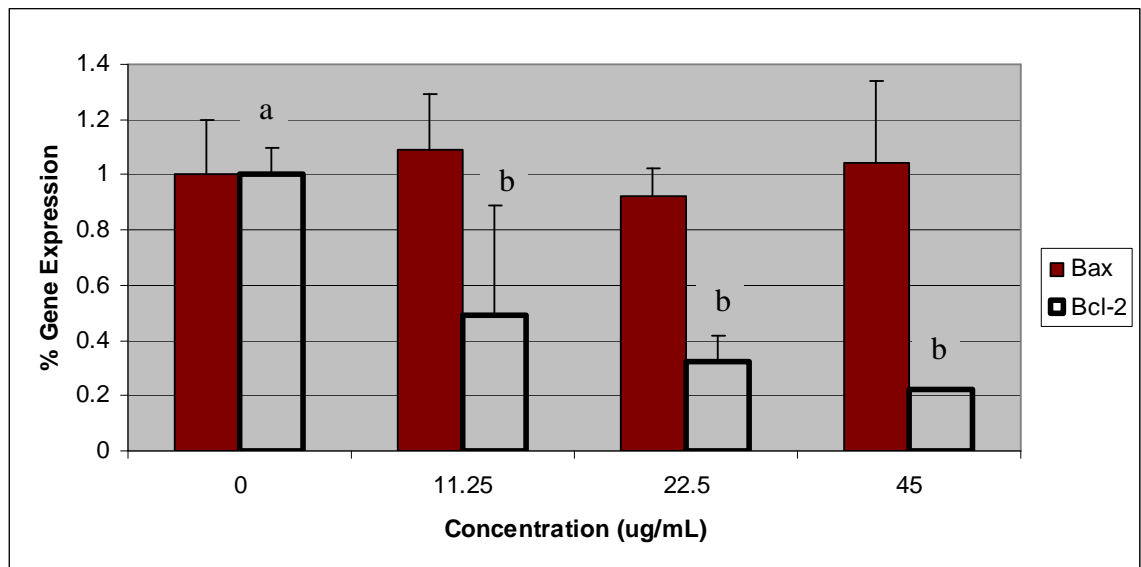
¹ Apoptotic gene expression was assessed by the real-time RT-PCR. Values are mean ± SD. Values that do not share the same letters were significantly (P < 0.05) different from each other.

Figure 7. The effect of resveratrol treatment for 48 hours on relative mRNA expression of apoptotic genes, Bax and Bcl-2, in MCF-7 human breast cancer cells ¹



¹ Apoptotic gene expression was assessed by the real-time RT-PCR. Values are mean \pm SD. Bars that do not share the same letters are significantly ($P < 0.05$) different from each other. P value for Bax = 0.0583; P value for Bcl-2 = 0.0024.

Figure 8. The effect of Rubaiyat grape extract treatment for 48 hours on relative mRNA expression of apoptotic genes, Bax and Bcl-2, in MCF-7 human breast cancer cells ¹



¹ Apoptotic gene expression was assessed by the real-time RT-PCR. Values are mean \pm SD. Bars that do not share the same letters are significantly ($P < 0.05$) different from each other. P value for Bax = 0.0583; P value for Bcl-2 = 0.0024.

CHAPTER V

DISCUSSION

The aim of this study is to compare the effect of Rubaiyat grape polyphenolic-rich extract to that of resveratrol in (a) inhibiting cell proliferation and (b) inducing apoptosis in the human breast cancer MCF-7 cell line. We chose the Rubaiyat grape because it is relatively high in polyphenol content and is a grape variety native to Oklahoma. The total phenolic content of the Rubayat grape extract used in our study, determined by the Folin-Ciocalteu method, was approximately 1140 $\mu\text{g/mL}$. For our study, we used the whole grapes for extracting the phenolic compounds. However, polyphenols are found in the skin, pulp, and seeds of the grape; as much as 60 to 70% are in the seeds, very low amounts in the pulp, and as much as 30% in the skin (Zhao et al., 1999). We did not attempt to extract the phenolic compounds from each of the components of grapes. Active constituents of grapes that may possess anti-carcinogenic properties are polyphenolic compounds such as flavonoids, anthocyanins, and stilbene derivatives (Nassiri-Asl and Hosseinzadeh, 2009). Flavan derivatives are common polyphenols in grapes and found as dimers, trimers, and oligomers in the seed and are commonly referred to as procyanidins or proanthocyanidins (Zhao et al., 1999).

Flavonoids are antioxidants present in the grape skin and seeds (Yilmaz and Toledo, 2004); these compounds are flavan-3-ols which are colorless and include epicatechin and catechin. The colored compounds are flavanones such as quercetin and red and blue anthocyanins (Shi et al, 2003). Anthocyanins are responsible for the blue, purple and red color of many plant tissues including grapes (Prior and Wu, 2006). Common anthocyanin compounds in grapes are glucosides, caffeoylglucosides, diglucosides of cyanidins, delphinidin, peonidin, petunidin, and malvidin (Wang et al., 2003). The major stilbene derivative found in grapes is resveratrol (Iriti and Faoro, 2006), which we used as our positive control. The anthocyanin and flavonoid of the Rubaiyat grape extract in our study was determined to be approximately 66 and 620 $\mu\text{g/mL}$, respectively. However, the amount of these active compounds present in grapes depends on several factors including the cultivar, year of production (climate), site of production (geographic origin, soil quality, and fertilization), and degree of maturation (Shi et al., 2003).

In our study we compared the effects of different doses of Rubaiyat grape extract to that of resveratrol in inhibiting human MCF-7 cell proliferation. We have found that higher doses of the extract are needed to inhibit MCF-7 proliferation in comparison to resveratrol. This is understandable as the concentration of the Rubaiyat grape extract used in the study was based on the total phenolic content of the grape extract. The identity of compounds in the extract and the molecular weights are unknown and so the Rubaiyat grape extract concentration was based on the total phenolic compounds equivalent to resveratrol. When comparing overall concentration with treatment with time, we found statistical significance ($P = 0.0078$) regardless of treatment type.

In our study, we used 11.25 µg/mL (50 µM) as the lowest and 228 µg/mL (1000 µM) as the highest concentrations of resveratrol in order to see dose-dependent effects. We observed that inhibition of cellular proliferation by resveratrol started at 11.25 µg/mL (50 µM) but marked inhibition was observed at 45 µg/mL (200 µM) and no additional benefit with 228 µg/mL (1000 µM). Similar to our study, some of the studies investigating anti-cancer properties of resveratrol have demonstrated inhibitory effects at 50 µM (Kim et al., 2004; Murias et al., 2008; Pozo-Guisado et al., 2002). For example, MCF-7 breast cancer cells treated with 50 µM resveratrol showed an accumulation of cells in the early phases of the cell cycle as compared to the controls which showed an increased amount of cells in the late and final phases of the cell cycle (Kim et al., 2004).

Although 11.25 µg/mL (50 µM) was shown to be effective, different concentrations of resveratrol have also been used in other studies. Murias et al. (2008) found that an analogue of resveratrol (3,3',4,4',5,5'-hexahydroxystilbene) was shown to inhibit cell growth in three breast cancer cell lines in a concentration-dependent manner. The three cell lines exhibited a concentration-dependent loss of mitochondrial potential while 50 µM of the analogue caused a time-dependent loss. In two of the cell lines (ZR-75-1 and T47D), caspase-3 and caspase-9 were detected while all three caspases (caspases -3, -8, and -9) showed activation in MDA-MB-231 cells. The results of this study demonstrated that apoptosis induced by the resveratrol analogue was dose-dependent and mitochondrial in origin as shown by the dissipation of mitochondrial membrane potential that is closely followed by caspase-9 activation (Murias et al, 2008).

It is interesting to note that the inhibition of cell proliferation may be affected by the type of isomer of resveratrol as shown by Basly and colleagues (2000). Their findings

demonstrated that the (*Z*)-isomer of resveratrol was less effective than the (*E*)-isomer on the inhibition of cellular proliferation in both MCF-7 and MVLN cell lines. Although we did not use specific isomers of resveratrol and Rubaiyat grape extract, there were still significant changes observed in cell proliferation that were concentration-dependent as seen in the literature (Basly et al., 2000, Murias et al., 2008). Anti-carcinogenic effects of the isomers of phenolic compounds isolated from Rubaiyat grapes needs to be explored.

Since our study used total phenolic extract, we cannot identify which of the phenolic compound(s) is responsible for the anti-carcinogenic effect of the Rubaiyat grape extract. The majority of the studies on grape polyphenols, except for studies on resveratrol, have utilized extracts of unknown composition. Red wine polyphenols have been shown to decrease *in vitro* breast cancer cell proliferation (Damianaki et al., 2000). Hakimuddin and colleagues (2004) examined the anti-proliferative activity of three different structural classes of flavonoids from wine on breast cancer cell lines, MCF-10A and MCF-7, and one normal human mammary epithelial cell line (HMEC). HMECs were most sensitive to the inhibition of the flavonoids as compared to MCF-10A and MCF-7 cell lines. The less hydrophobic fraction (the crude fraction) of the wine showed similar inhibitory effects on all three cell lines while the more hydrophobic fraction showed the most inhibition of MCF-7 cancer cell line proliferation but not the other two cell lines. The authors suggest that these results may be attributed to flavonoid fractions and growth inhibition is highly dependent on flavonoid concentration. Another study (Faria et al., 2006) evaluated the effects of procyanidin fractions on MCF-7 human breast carcinoma cells. Catechin fractions (30 and 60 µg/mL) caused a decrease in cell

viability, inhibited cell proliferation of MCF-7 cells, and exhibited higher antioxidant activity. The authors suggest that the antioxidant activity of procyanidins may be partly responsible for the effect on cell proliferation (Faria et al., 2006). Singletary et al. (2007) examined the effects of anthocyanin-rich extract from Concord grapes and anthocyanin delphinidin on human breast epithelial cells with DNA damage from an environmental carcinogen, benzo[a]pyrene (BP). Concord grape extract (CGE) inhibited BP-induced DNA adduct formation in the MCF-10F cell line. These findings indicate that in chemically induced breast carcinogenesis, CGE and select constituent anthocyanins may be considered as potential blocking agents (Singletary et al., 2007). Overall, the findings of these studies indicate that several components in grapes may exhibit anti-carcinogenic properties.

In addition to the inhibition of cellular proliferation, we also investigated the apoptotic effects of Rubaiyat grape extract and resveratrol. Cells were treated with different concentrations of Rubaiyat grape extract or resveratrol for 48 or 72 hours. The treatment was considered to induce apoptosis if cells were far less numerous and misshapen, smaller in size, and possibly indicating membrane blebbing. Grape extract and resveratrol treatment showed similar apoptotic results at their highest treatment concentration (228 $\mu\text{g/mL}$). Rubaiyat grape extract at 228 $\mu\text{g/mL}$ showed the most marked induction of apoptosis on human MCF-7 breast cancer cells compared to the control. Resveratrol-treated cells showed these same characteristics in a dose-dependent manner and were observed starting at the lowest concentration (11.25 $\mu\text{g/mL}$ = 50 μM concentration) and were most marked at 228 $\mu\text{g/mL}$ (1000 μM) concentration. Apoptosis induction was most prominent at 72 hours for both treatments in comparison to 48 hours.

Our findings indicate that apoptosis induction is both concentration- and time-dependent with the highest concentration of each treatment showing most prominent apoptotic characteristics. However, all these data analyses need to be interpreted with caution as apoptosis visualization was done using the naked eye and not by a quantitative method.

The mechanism by which resveratrol and Rubaiyat grape extract exert anti-carcinogenic effects is not clear at this time. Literature reports that growth suppression by resveratrol is the result of mid to late cell cycle arrest (Joe et al., 2002) and modulation of the apoptosis cascade (up-regulation of proapoptotic proteins, down-regulation of antiapoptotic proteins, and activation of caspase-3) (Nakagawa et al., 2001). Additionally, exposure of MCF-7 human breast cancer cells to resveratrol for 48 hours resulted in a dose-dependent cell cycle arrest and apoptosis (Kim et al., 2004). It was also observed that Bcl-2 protein content, a pro-apoptotic protein, is inversely proportional to the dose of resveratrol (Pozo-Guisado et al., 2005), and resveratrol was unable to induce caspase-3 and caspase-8 activity, pro-apoptotic enzymes, in the MCF-7 breast cancer cell line (Pozo-Guisado et al., 2005), at concentrations similar to the ones used in our study.

We also examined the effects of different concentrations of resveratrol and Rubaiyat grape extract on the gene expression of anti- and pro-apoptotic markers, Bcl-2 and Bax, respectively. Rubaiyat grape extract treatment starting at 11.25 $\mu\text{g/mL}$ and only 45 $\mu\text{g/mL}$ of resveratrol caused a significant reduction of Bcl-2 gene expression. Jourdain and Martinou (2009) reported that the Bcl-2 family is pro-apoptotic proteins which permeabilize the mitochondria during apoptosis by integrating and conveying death signals to the mitochondria in order to cause the release of other proteins and enzymes to continue with the apoptotic process. These proteins act closely together signaling one

another in a cascade to promote the apoptosis process (Jourdain and Martinou, 2009 and Tsujimoto, 2003). Similar studies determined that induction of cell death by resveratrol was shown to be associated to Bcl-2 phosphorylation (Tinhofer et al., 2001), Bax mitochondrial translocation (Mahyar-Roemer, Kohler, and Roemer, 2002), and inhibition of AKT and FAK activity (Brownson et al., 2002). Additionally, reduction of Bcl-2 gene expression was similar to that observed by Mantena et al (2006) with grape seed procyanidins treatment of mouse metastatic breast cancer cell line (4T1).

Both resveratrol and Rubaiyat grape extract treatments had no significant effect on pro-apoptotic Bax expression. We do not know the reason why we did not see treatment effects on Bax gene expression since we observed some changes characteristic of apoptosis.

The concentrations of resveratrol used in our study were similar to those used in other studies (Joe et al., 2002, Nakagawa et al., 2001, Kim et al., 2004, Pozo-Guisado et al., 2005). Our results on the effects of resveratrol on inhibition of cellular proliferation (Joe et al., 2002, Nakagawa et al., 2001, Kim et al., 2004, Pozo-Guisado et al., 2005) and apoptosis induction (Kim et al., 2004, Nakagawa et al., 2001, Pozo-Guisado et al., 2005) were similar to those of other investigators. However, our findings on the gene expression of Bax and Bcl-2 genes were different from others. Other studies show increased expression of Bax and decreased expression of Bcl-2 with increasing concentrations of resveratrol (Kim et al., 2004, Nakagawa et al., 2001, Pozo-Guisado et al., 2005). We found that resveratrol had no significant effect on pro-apoptotic Bax gene expression and anti-apoptotic Bcl-2 gene expression was only significantly decreased at

the highest concentration (45 µg/mL which is equivalent to 200 µM) of resveratrol treatment.

In summary, Rubaiyat grape extract inhibited cell proliferation and induced apoptosis in the MCF-7 cell line. Since other components are present in the Rubaiyat grape extract, higher concentrations of the extract were needed to inhibit cell proliferation and induce apoptosis in this cell line. The anti-carcinogenic property of the Rubaiyat grape extract may be attributed in part to the inhibition of Bcl-2 but not Bax. However, these are the only genes that were examined and other genes might be affected more by Rubaiyat grape extract.

Recommendations for future studies

Further exploration of other apoptotic markers is warranted in order to better understand how polyphenols affect apoptosis in human MCF-7 breast cancer cells. One step further is to assess the effects of the Rubaiyat grape extract in an animal model. For example, Mantena et al. (2006) gave immunocompetent Balb/c mice a diet consisting of 0.2% and 0.5% w/w grape seed procyanidins (GSPs). Dietary GSPs significantly inhibited the growth of subcutaneous tumors and increased survival rate in immune competent Balb/c mice, inoculated with 4T1 breast cancer cells. GSP also inhibited metastasis of the 4T1 cells from the primary tumor site to the lungs, decreased expression of antiapoptotic Bcl-2, increased Bax, cytochrome c, and Apaf-1 expression as well as activate caspase-3 in the tumors compared to control mice (Mantena et al., 2006). Whether Rubaiyat grape extract exerts similar effects needs to be explored.

It may also be possible in future studies to better understand the use of resveratrol and grape extracts in the reduction of estrogen-related breast cancers. *In vitro*, resveratrol was shown to inhibit other known key regulators of cell proliferation such as PKC (Stewart et al., 1999), ribonucleotide reductase therefore interfering with DNA synthesis in proliferating cancer cells (Fontecave et al., 1998), and both the activity and transcription of COX2 (Subbaramaiah et al., 1998). These are other important regulators of cellular proliferation that warrant further studies using resveratrol and Rubaiyat grape extract in human MCF-7 cells to better understand the mechanism behind inhibition of proliferation and induction of apoptosis.

Also, further research in the area of Rubaiyat grape extract and resveratrol is needed to identify a safe dosage to consume on a regular basis for supplementation. Moreover, the amount of whole grape that needs to be consumed for anti-cancer effects needs to also be explored. The findings from this study are to be taken with caution as they do not indicate a safe dosage of extract or resveratrol or amount of whole grape to be consumed. More *in vitro* and *in vivo* studies need to be conducted in order to determine the proper dosage or consumption recommendations in which to see beneficial effects.

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VITA

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Thesis: THE EFFECTS OF THE EXTRACT OF RUBAIYAT, AN OKLAHOMA
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Scope and Method of Study: Breast cancer is the most frequently diagnosed cancer in women and ranks second as cause of cancer death in women. Recent data show that some dietary antioxidants may have potential benefits in cancer therapy. Studies show that resveratrol is structurally similar to estrogen and is therefore implicated for use as an anticancer and therapeutic agent in breast cancer. This study compared the effects of the extract of the Rubaiyat grape to that of resveratrol in inhibiting human breast cancer MCF-7 cell proliferation and inducing apoptosis. Rubaiyat grape extract used in this study contains 1141 $\mu\text{g/mL}$ total polyphenol, 620 $\mu\text{g/mL}$ flavonoid, and 66 $\mu\text{g/mL}$ anthocyanin.

Findings and Conclusions: Rubaiyat grape extract inhibited cell proliferation and induced apoptosis in human MCF-7 breast cancer cells. Since other components are present in the Rubaiyat grape extract, higher concentrations of the extract were needed to inhibit cell proliferation and induce apoptosis in this cell line. The anti-carcinogenic property of the Rubaiyat grape extract may be attributed in part to the inhibition of Bcl-2 but not Bax. However, these are the only genes that were examined and other genes might be affected more by Rubaiyat grape extract.

ADVISER'S APPROVAL: Dr. Edralin A. Lucas
